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(71) Applicant *(for all designated States except US)*: **PROTEIN DESIGN LABS INC.** [US/US]; 34801 Campus Drive, Fremont, CA 94555 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **TSO, Yun, J.** [US/US]; 445 Oak Grove Avenue, #16, Menlo Park, CA 94025 (US). **MCPHATE GREEN, Jennifer** [US/US]; 3631 Hillcrest Drive, Belmont, CA 94402 (US).

(74) Agents: **HALLUIN, Albert** et al.; HOWREY SIMON ARNOLD & WHITE, LLP, 301 Ravenswood Avenue, Box 34, Menlo Park, CA 94025 (US).

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(54) Title: IDENTIFYING ANTI-TUMOR TARGETS OR AGENTS BY LIPID RAFT IMMUNIZATION AND PROTEOMICS

(57) Abstract: The present invention is directed to a method for identifying anti-tumor targets by examining lipid rafts. It provides for a method for identifying anti-tumor targets by lipid raft proteomics and a method for identifying anti-tumor agents by lipid rafts immunization. It also provides for hybridomas produced by the method of identifying anti-tumor agents by lipid raft immunization, and antibodies produced by the hybridomas. It also provides for the anti-tumor targets or agents identified by the methods in the present invention.

IDENTIFYING ANTI-TUMOR TARGETS OR AGENTS BY LIPID RAFT IMMUNIZATION AND PROTEOMICS

Field of the Invention

This invention concerns methods for identifying anti-tumor targets or agents by lipid raft proteomics or by lipid raft immunization.

Background of the Invention

Lipid rafts are regions on the plasma membrane that have a different composition of lipids than the surrounding plasma membrane. They are enriched in signaling molecules and can change their size and composition in response to intra- or extracellular stimuli (Simons, K., et al., Nature Reviews / Molecular Cell Biology: Vol. 1 pp 31-39 (2000)). This action favors specific protein-protein interactions, resulting in the activation of signaling cascades. The most important role of rafts at the cell surface is their function in signal transduction. It has been shown that growth factor receptors and sensor molecules migrate to lipid rafts after ligand binding or cross-linking. It is known that growth factor receptors are closely related to tumor formation. Therefore lipid rafts are a good source of tumor-associated antigens. Accordingly, the current invention uses lipid rafts instead of entire cell membranes in the search for the cancer-related molecules (tumor targest).

Summary of the Invention

The present invention provides a method for identifying a tumor target comprising examining a lipid raft, wherein said lipid raft is derived from a tumor cell.

Preferably, said examining comprises: isolating lipid rafts from a tumor cell and a normal cell; comparing the lipid raft protein expressions of said tumor cell and said normal cell; isolating a molecule that is differentially expressed in said tumor cell. More preferably, said method further comprises: identifying partial or full amino acid sequence of said molecule, or partial or full nucleic acid sequence encoding said molecule.

Preferably, said tumor target is a prostate tumor target, wherein said tumor cell is a prostate tumor cell and said normal cell is a normal prostate cell.

The present invention provides a method for generating an antibody against a tumor target associated with a type of tumor cells, comprising: isolating lipid rafts from said type of tumor cells; and immunizing an animal host by said lipid rafts. producing

hybridomas from the immunized animal host selecting said monoclonal antibodies; and purifying said selected antibodies

The present invention provide for an isolated lipid raft derived from a prostate cancer cell, wherein said isolated lipid raft comprising a polypeptide that is differentially expressed in a prostate tumor cell. Preferably, said polypeptide is selected from the group consisting of PMSA, CD10, Trop-1, ATP synthase, NCAM2, and CD222.

The present invention provides an isolated monoclonal antibody that binds to the isolated lipid raft, wherein said monoclonal antibody binds to or neutralizes PMSA, CD10, Trop-1, ATP synthase, NCAM2, or CD222.

Brief Description of the Drawings

Figure 1. Comparison of lipid rafts prepared from normal prostate cells and three prostate carcinoma cell lines, DU 145, LNCaP, and PC-3. Equal amounts of protein from lipid raft preparations were separated by SDS-PAGE and then visualized by silver staining. Differentially expressed proteins are marked.

Figure 2. Comparison of lipid rafts prepared from LNCaP and normal prostate cells by 2-dimensional electrophoresis. Equal amounts of protein from lipid raft preparations were separated by 2-dimensional electrophoresis and then visualized by silver staining. Protein spots that are present in the LNCaP sample, but not the normal prostate sample are denoted with arrows. Protein spots that have been identified by peptide mass profiling are labeled with numbers (see Table 1).

Figure 3. ATP synthase is present in lipid rafts preparations from prostate cancer cell lines, but not from normal prostate cells. Equal amounts of lipid raft proteins prepared from normal prostate cells and three prostate carcinoma cell lines (LNCaP, DU 145, and PC-3) were separated by SDS-PAGE and then electrotransferred onto a PVDF membrane. Western blotting was performed using an antibody specific for ATP synthase followed by HRP-conjugated goat anti-mouse IgG. The membrane was developed using enhanced chemiluminescence.

Figure 4. ATP synthase is present in rafts prepared from many different cancer cell lines. Equal amounts of lipid raft proteins prepared from various cancer cell lines (BeWo, Colo205, HT-29, JEG-3, KG-1, LS 180, MCF-7, LNCaP, NCI-H292, PANC-1, RT-4, and THP-1) were separated by SDS-PAGE and then electrotransferred onto a PVDF membrane. Western blotting was performed as described in Figure 3.

Figure 5. Flow cytometric analysis of ATP synthase cell surface expression. LNCaP cells were resuspended in 100 μL PBS/5% fetal calf serum with 1 μg anti-ATP synthase

(middle) or anti-Trop-1 (EpCAM) (right). Cells were also stained with a negative control antibody (left). Cells were washed and bound antibody was detected with PE-conjugated goat anti-mouse IgG. Cells were then analyzed by flow cytometer.

Figure 6. Flow cytometric analysis of ATP synthase expression in the AML cell line THP-1. THP-1 cells were resuspended in 100 μL PBS/5% fetal calf serum with 1 μg anti-ATP synthase α subunit (middle) or β subunit (right). Cells were also stained with a negative control antibody (left). Cells were washed and bound antibody was detected with PE-conjugated goat anti-mouse IgG. Cells were then analyzed by flow cytometer.

Figure 7. ATP synthase is localized to the cell surface of LNCaP prostate carcinoma as visualized by immunofluorescence. LNCaP cells, grown on glass coverslips, were stained with antibodies specific for ATP synthase (top) or Trop-1 (EpCAM) (bottom). Bound antibody was detected with Alexa 488-conjugated goat anti-mouse IgG. Coverslips were mounted onto slides and examined with a Nikon Optiphot 2 microscope and photographed. No staining is observed on cells that have been stained with the secondary antibody alone (data not shown).

Figure 8. Anti-ATP synthase inhibits LNCaP cell proliferation. LNCaP cells (20,000 cells/well) were plated into a 96 well tissue culture plate. After cells were allowed to grow undisturbed for two days, antibodies (5 μg/ml anti-ATP synthase, anti-Trop-1 (EpCAM) (323/A3), or anti-MHC class II (Mu1D10)) were added and incubated with the cells for 24 hours. AlamarBlue reagent was added to assess cell proliferation. Fluorescence was detected at λex=530nm, λem=590 nm. Data are expressed as the mean +/- SEM of 4 replicates.

Figure 9. Anti-ATP synthase inhibits LNCaP colony formation in soft agar. LNCaP cells were plated in soft agar and treated with anti-ATP synthase or anti-Trop-1 (EpCAM) (5 μg/ml) for up to 20 days. Colonies were counted under an inverted phase-contrast microscope and a group of 5 or more cells were counted as a colony.

Figure 10. Anti-ATP synthase induces apoptosis in THP-1 cells. THP-1 cells were treated with anti-ATP synthase or anti-Trop-1 (EpCAM) (5 μg/mL) for 24 hours. Cells were then harvested at the indicated times after the induction of apoptosis and were stained with FITC-conjugated annexin V and propidium iodide. Flow cytometry was used to assess percentage of apoptosis (annexin V^+ and propidium $iodide^{-/+}$ cells).

Figure 11. Comparison of lipid rafts from various cancer cell lines. Equal amounts of protein from lipid raft preparations were separated by SDS-PAGE and then visualized by silver staining.

Figure 12. Flow chart summarizing how the tumor-specific hybridomas were obtained from the LNCaP lipid raft immunization.

Figure 13. Antigen grouping by immunoprecipitation. ^{125}I labeled LNCaP lysate was incubated individually with 20 hybridoma supernatants (see Table 2). Antibody-antigen complexes were captured by Gamma Bind Plus Sepharose and analyzed by SDS-PAGE. Lane 1, P1-42; Lane 2, P2-23; Lane 3, P3-53; Lane 4, P4-79; Lane 5, P6-49; Lane 6, P9-65; Lane 7, P8-2; Lane 8, P8-11; Lane 9, P8-14; Lane 10, P8-35; Lane 11, P8-74; Lane 12, P9-32; Lane 13, P9-64; Lane 14, P10-2; Lane 15, P10-28, Lane 16, P10-29; Lane 17, P10-62; Lane 18, P10-70; Lane 19, P10-82; and Lane 20, P12-22. Molecular weight standards (MW) are in kD.

Figure 14. Anti-NCAM2 inhibits LNCaP cell proliferation. LNCaP cells (20,000 cells/well) were plated into a 96 well tissue culture plate. After cells were allowed to grow undisturbed for two days, 4 different NCAM2-specific antibodies (5 μg/ml) were added and incubated with the cells for 24 hours. AlamarBlue reagent was added to assess cell proliferation. Fluorescence was detected at λex=530nm, λem=590 nm. Data are expressed as the mean +/- SEM of 4 replicates.

Figure 15. Anti-NCAM2 inhibits LNCaP colony formation in soft agar. LNCaP cells were plated in soft agar and treated with 4 different NCAM2-specific antibodies (5 μg/ml) for up to 20 days. Colonies were counted under an inverted phase-contrast microscope and a group of 10 or more cells were counted as a colony.

Figure 16. Antigen grouping by immunoprecipitation. ^{125}I labeled LNCaP lysate (Lanes 1-10) or Panc-1 lysate (Lanes 11-16) were incubated individually with 16 hybridoma supernatants (see Tables 2 and 3) that showed broad specficity against many cancer cell lines (see Table 3). Antibody-antigen complexes were captured by Gamma Bind Plus Sepharose and analyzed by SDS-PAGE. Lane 1, P12-27; Lane 2, P11-65; Lane 3, P8-83; Lane 4, P8-32; Lane 5, P8-20; Lane 6, P7-69; Lane 7, P4-48; Lane 8, P3-28; Lane 9, P2-68; Lane 10, P1-95; Lane 11, P11-93; Lane 12, P11-85; Lane 13, P11-57; Lane 14, P11-49; Lane 15, P8-35, Lane 16, P8-11. Molecular weight standards (MW) are in kD.

Figure 17. Flow chart summarizing how the tumor-specific hybridomas were obtained from the KG-1 lipid raft immunization.

Figure 18. Antigen grouping by immunoprecipitation. (A). ^{125}I labeled KG-1 lysate was incubated individually with 36 hybridoma supernatants (see Table 4). Antibody-antigen complexes were captured by Gamma Bind Plus Sepharose and analyzed by SDS-PAGE. Lane 1, K1-34; Lane 2, K1-47; Lane 3, K1-79; Lane 4, K1-95; Lane 5, K1-97; Lane 6, K2-109; Lane 7, K2-124; Lane 8, K2-127; Lane 9, K2-167; Lane 10, K5-37; Lane 11, K5-71; Lane 12, K6-98; Lane 13, K6-103; Lane 14, K6-114; Lane 15, K6-121, Lane 16, K6-149. Lane 17, K6-150; Lane 18, K6-175; Lane 19, K6-179; Lane 20, K7-196; Lane 21, K7-270; Lane 22, K7-275; Lane 23, K8-335; Lane 24, K8-343; Lane 25, K8-355; Lane 26, K8-364; Lane 27, K8-365; Lane 28, K9-3; Lane 29, K9-64; Lane 30, K9-92; Lane 31, K11-230, Lane 32, K11-272, Lane 33, K11-280, Lane 34, K11-282, Lane 35, K12-328, Lane 36, K12-360. Molecular weight standards (MW) are in kD. (B). Biotinylated KG-1 lysate was incubated individually with 15 hybridoma supernatants that failed to identify the molecular weight of their antigens in the experiment shown in panel (A). Antibody-antigen complexes were captured by Gamma Bind Plus Sepharose, analyzed by SDS-PAGE. Lane 1, K8-364; Lane 2, K8-365; Lane 3, K9-92; Lane 4, K11-272; Lane 5, 11-280; Lane 6, K6-150; Lane 7, K6-149; Lane 8, K2-109; Lane 9, K2-127; Lane 10, K5-71; Lane 11, K6-103; Lane 12, K6-114; Lane 13, K6-121; Lane 14, K9-64; Lane 15, K7-196. Molecular weight standards (MW) are in kD.

Figure 19. Nucleotide sequence and deduced amino acid sequence of the heavy chain variable region (VH) of K8-355 (anti-HLA-DR). The signal peptide is in italic, the three complementarity determining regions (CDRs) are underlined, and the first NH2-terminal amino acid residue of the matured heavy chain (Mouse IgG1) is in bold. SEQ ID NO: 1 is the amino acid sequence of the heavy chain variable region (VH) of K8-355 (anti-HLA-DR) (signaling peptide is not included).

Figure 20. Nucleotide sequence and deduced amino acid sequence of the light chain variable region (VH) of K8-355 (anti-HLA-DR). The signal peptide is in italic, the three complementarity determining regions (CDRs) are underlined, and the first NH2-terminal amino acid residue of the matured light chain (mouse kappa) is in bold. SEQ ID NO: 2 is the amino acid sequence of the light chain variable region (VL) of K8-355 (anti-HLA-DR) (signaling peptide is not included).

Figure 21. K8-355 (anti-HLA-DR) induces apoptosis in Raji and Daudi cells. Raji or Daudi cells were treated with K8-355 (5 μg/mL) for 24 hours. Cells were then harvested

at the indicated times after the induction of apoptosis and were stained with FITC-conjugated annexin V and propidium iodide. Flow cytometry was used to assess percentage of apoptosis (annexin V^+ and propidium $iodide^{-/+}$ cells).

Figure 22. Table 1 shows five identified proteins from LNCaP lipid raft 2-D samples by peptide mass profiling.

Figure 23. Table 2 shows the reactivity profiles of 20 anti-LNCaP lipid raft hybridomas that showed limited binding to other cancer cell lines

Figure 24. Table 3 shows the reactivity profiles of 1 anti-LNCaP lipid raft hybridomas that showed broad binding to other cancer cell lines.

Figure 25. Table 4 shows the reactivity profiles of 36 anti-KG-1 lipid raft hybridomas that showed apoptosis-inducing activity.

Detailed Description of the Preferred Embodiments

As used herein, the term "differentially expressed" means that a protein or polypeptide is expressed at a higher level, for instance, 20% more, preferably 50% more, more preferably, 80% more, in a cell type that is cancerous (tumor cell), than that in said cell type that is non-cancerous (normal cell).

The term "tumor target" and "anti-tumor target" are used interchangeable herein and refers to any molecules in correlation with the occurrence or existence of a tumor or a tumor cell. Preferably, a tumor target is expressed at a higher level in a type of tumor cell than that in the normal cells. The term "anti-tumor agent" refers to any molecules that can inhibit tumor or cancer cell growth. The anti-tumor agent may reduce the growth rate or the size of tumor cells, or inhibit or prevent proliferation or migration of tumor cells. It may inhibit the colony formation of cancer cells due to the anchorage-independent growth. Preferably, such an inhibition at the cellular level can reduce the tumor size, deter or reverse the growth of a tumor, reduce the aggressiveness of a tumor, or prevent or inhibit tumor metastasis. Preferably anti-tumor agent is an inhibitor of a tumor targets. The inhibitor can be an antibody against the tumor targets, or a molecule inhibiting the activities of the tumor targets, or a molecule down-regulating the expression of tumor targets, or a molecule down-regulating the transcription of DNA encoding the tumor

targets, or an anti-sense nucleic acid sequence of partial or full nucleic acid sequence encoding the tumor target. More preferably, it is an antibody against a tumor target associated with a type of tumor.

The term "lipid raft" refers to a lipid raft or a portion thereof in a clustered state or a non-clustered state, including "lipid raft", "clustered lipid rafts", and "DRM", each of which has been described in detail in Simons, K., et al., Nature Reviews / Molecular Cell Biology: Vol. 1 pp 31-39 (2000). In particular, "lipid raft" contains a given set of proteins that can change size and composition in response to intra- or extracellular stimuli. This favors specific protein-protein interactions, resulting in the activation of signally cascade. Sometimes, the lipid rafts may be clustered together. It has been reported that clustering is used both artificially and physiologically to trigger signally cascades. DRMs (detergent-resistant membranes) are the rafts that remain insoluble after treatment on ice with detergents . They are believed to be non-native aggregated rafts.

The term "apoptosis", "apoptotic cell death" or "programmed cell death" as used herein refers to any cell death that results from the complex cascade of cellular events that occur at specific stages of cellular differentiation and in response to specific stimuli. Apoptotic cell death is characterized by condensation of the cytoplasm and nucleus of dying cells.

The term "colony formation" refers to the number of colonies formed due to the inhibition of the anchorage-independent cell growth. Various methods known in the art can be used to measure the colony formation such as counting the number of tumor cell colonies formed.

The term "antibody" or "immunoglobulin" is intended to encompass both polyclonal and monoclonal antibodies. The preferred antibody is a monoclonal antibody reactive with the antigen. The term "antibody" is also intended to encompass mixtures of more than one antibody reactive with the antigen (e.g., a cocktail of different types of monoclonal antibodies reactive with the antigen). The term "antibody" is further intended to encompass whole antibodies, biologically functional fragments thereof, single-chain antibodies, and genetically altered antibodies such as chimeric antibodies comprising portions from more than one species, bifunctional antibodies, antibody conjugates, humanized and human antibodies. Biologically functional antibody fragments, which can also be used, are those peptide fragments derived from an antibody that are sufficient for binding to the antigen.

By "a pharmaceutically effective" amount of a drug or pharmacologically active agent or pharmaceutical formulation is meant a nontoxic but sufficient amount of the drug, agent or formulation to provide the desired effect.

A "subject," "individual" or "patient" is used interchangeably herein, which refers to a vertebrate, preferably a mammal, more preferably a human.

The term "genetically altered antibodies" means antibodies wherein the amino acid sequence has been varied from that of a native antibody. Because of the relevance of recombinant DNA techniques to this invention, one need not be confined to the sequences of amino acids found in natural antibodies; antibodies can be redesigned to obtain desired characteristics. The possible variations are many and range from the changing of just one or a few amino acids to the complete redesign of, for example, the variable or constant region. Changes in the constant region will, in general, be made in order to improve or alter characteristics, such as complement fixation, interaction with membranes and other effector functions. Changes in the variable region will be made in order to improve the antigen binding characteristics.

The term "humanized antibody" or "humanized immunoglobulin" refers to an immunoglobulin comprising a human framework, at least one and preferably all complimentarity determining regions (CDRs) from a non-human antibody, and in which any constant region present is substantially identical to a human immunoglobulin constant region, i.e., at least about 85-90%, preferably at least 95% identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDRs, are substantially identical to corresponding parts of one or more native human immunoglobulin sequences. See, e.g.Queen et al., U.S. Patent Nos: 5,5301,101; 5,585,089; 5,693,762; and 6,180,370 (each of which is incorporated by reference in its entirety).

The term "chimeric antibody" refers to an antibody in which the constant region comes from an antibody of one species (typically human) and the variable region comes from an antibody of another species (typically rodent).

I. Lipid rafts

The present invention provides for methods for identifying anti-tumor agents (tumor targets) or anti-tumor targets by examining lipid raft, preferably by lipid raft proteomics or lipid raft immunization.

The methods of identifying anti-tumor targets or agents in the present invention comprise examining lipid rafts. The methods can be used in search for anti-tumor targets in various cancer types, including but not limited to cancers of the brain, breast, cervix,

bladder, colon, head & neck, kidney, liver, lung, non-small cell lung, lymphoid system, pancreas, prostate, ovary, stomach, uterus, medulloblastoma, melanoma, mesothelioma sarcoma, and other like cancers.

Preferably, the methods for identifying anti-tumor targets or agents further comprises preparing and examining the lipid rafts from a certain type of cells, wherein the type of cells can be of normal cells or cancer cells from various tissues, including but not limited to blood, brain, breast, cervix, bladder, colon, head & neck, kidney, liver, lung, lymphoid system, non-small cell lung, ovary, pancreas, prostate, stomach, uterus, Medulloblastoma, melanoma, mesothelioma, sarcoma and other like tissues.

More preferably, the lipid rafts can be isolated from established cancer cell lines and studied in search for anti-tumor agents. The cell lines include, but are not limited to, LNCaP (prostate cancer), DU 145 (prostate cancer), PC-3 (prostate cancer), PANC-1 (pancreas cancer), RT4 (bladder cancer), HT-29 (colon cancer), NCI-H292 (lung cancer), T47D (breast cancer), Hep G2 (liver cancer), and NIH:OVCAR-3 (ovary cancer), AML cells, and other like tumor cell lines.

The lipid rafts can be isolated by the methods known in the art, such as the method described in Green et al, J. *Cell Biol.* 146, 673-682 (1999). In particular, cells are lysed and add to a sucrose solution to form a sucrose step-gradient. The gradients are then centrifuged, and the lipid rafts float to a fraction of the gradients. That fraction is then isolated and concentrated.

The isolated lipid rafts can then be used for the search of tumor targets or anti-tumor agents by lipid raft proteomics or lipid raft immunization.

II. Lipid Raft Proteomics

The method for identifying anti-tumor targets by lipid raft proteomics comprises identifying proteins that are differentially expressed in a type of cancer cells. In particular, protein expressions of lipid rafts from tumor cells and normal cells are compared. The molecules that are differentially expressed in the lipid rafts of tumor cells will be isolated, analyzed, and recognized as tumor targets. The anti-tumor agents can then be identified by selecting the inhibitors of the tumor targets.

In particular, the present invention provides a method for identifying a tumor target comprising examining a lipid raft, wherein said lipid raft is derived from a tumor cell or a normal cell. Typically, said examining comprises: isolating lipid rafts from a tumor cell and a normal cells; comparing the lipid raft protein expressions of said tumor

cell and said normal cell; isolating a protein that is differentially expressed in said tumor cell. Preferably, said tumor target is a prostate tumor target, said tumor cell is a prostate tumor cell and said normal cell is a normal prostate cell. More preferably, the prostate cancer cells are the cells of prostate cancer cell lines, including, but not limited to, LNCaP, DU145, and PC-3. Preferably, the prostate tumor target is ATP synthase

In addition, the method can further comprise: identifying partial or full amino acid sequence of the isolated protein, or partial or full nucleic acid sequence encoding said isolated protein. The most commonly used method of identifying amino acid sequence is N-terminal sequencing. The experimental procedure of N-terminal amino acid sequencing is disclosed in the Examples of the present application.

To compare the lipid raft proteins expressions, the proteins contained in the isolated lipid rafts may need to be separated. Various methods known in the art may be used to separate the proteins of the lipid rafts, such as one-dimensional electrophoresis or two-dimensional electrophoresis. The separated proteins may then be visualized by any standard methods known in the art, including, but not limited to, silver staining. The experimental procedures of performing one or two-dimensional protein electrophoresis and silver staining are disclosed in the Examples of the present application.

Preferably, the method of identifying anti-tumor targets by lipid raft proteomics comprises: isolating lipid rafts; separating the lipid rafts by means of electrophoresis or liquid chromatography, so that individual protein bands are separated from each other. comparing the protein expressions of said lipid rafts from cancer cells and from normal cells; isolating a protein band that is differentially expressed in cancer cells; and; identifying partial or full amino acid sequence of the protein, or partial or full nucleic acid sequence encoding the protein.

Preferably, the present invention provides for a method for identifying anti-tumor agents comprising selecting an inhibitor of said isolated protein. The inhibitor can be an antibody against said protein, or a molecule inhibiting the activities of said protein, or a molecule down-regulating the expression of said protein, or a molecule down-regulating the transcription of DNA encoding said protein, or an anti-sense nucleic acid sequence of partial or full nucleic acid sequence encoding said protein.

More preferably, the anti-tumor activity of said anti-tumor agents may be further verified. Once a tumor-related protein (tumor target) is identified, antibodies recognizing the protein can be generated and a Western-blot analysis can be performed using the antibodies to confirm that the protein is indeed differentially expressed in the cancer cells.

Anti-tumor agents may be created to inhibit the expression or function of that cancer-related protein. The Anti-tumor activity of the anti-tumor agent can be measured by performing a variety of experiments, including but not limited to, *in vitro* cell proliferation assay. The *in vivo* anti-tumor activity of the anti-tumor agents can be further verified by testing the efficacy using animal xenograft models. Cell adhesion and migration assays can be performed to evaluate the inhibition of adhesion and spreading of tumor cells by the anti-tumor agents. The experimental details of cell proliferation assay, xenograft model, and cell adhesion and migration assays are described in the Examples of the present application.

Preferably, the method for lipid raft proteomics can be used for the purpose of identifying anti-tumor targets or agents exclusively for the treatment of a particular type or subtype of cancer. Molecules (tumor targets) relating only to one type of cancer or a subtype of cancer can be identified by comparing the protein expressions of lipid rafts isolated from that type of cancer cells (for example prostate cancer cells), from other cancer cells, and from normal cells. The proteins only expressed in that type of cancer cells (for example, prostate cancer cells), but not to other type of cancers are further isolated and identified as the tumor targets of certain type or subtype of cancer according to methods provided in the present invention. The example of cancer subtype includes, but is not limited to, androgen-dependent prostate cancer and androgen-independent prostate cancer.

The present invention provides for an isolated protein, wherein said isolated protein is expressed at higher rate in a cell type that is cancerous than said cell type that is non-cancerous.

The present invention provides for an inhibitor of said isolated protein.

Preferably, the inhibitor is an antibody against said isolated protein.

II. Lipid Raft Immunization

The present invention provides for a method of identifying tumor targets or anti-tumor agents by lipid raft immunization. Different from lipid raft proteomics, lipid raft immunization produces monoclonal antibodies against lipid rafts derived from a type of tumor cells. Such monoclonal antibodies can be directly used as anti-tumor agents after the verification of their anti-tumor activities. The antigens that bind to such monoclonal antibodies are then identified. If the antigens are in correlation with tumor cells, such as differentially expressed in certain type of tumor cells, these antigens are then recognized

as the tumor targets for that type of tumor, and the monoclonal antibodies are recognized as anti-tumor agents.

The present invention provide for a method for identifying anti-tumor agents such as antibodies against a tumor target associated with a type of tumor comprising isolating lipid rafts from said type of tumor cells; immunizing an animal with the isolated lipid rafts. Lipid raft preparation from cancer cells may be injected into an appropriate host animal, such as cow, horse, goat, rat, mouse, hamster, or macaque monkey, etc. The immunization may be boosted by multiple sequential injections. A suggested protocol includes, but is not limited to, injection of 50 μg lipid raft proteins on Day 7 and Day 14 after the initial immunization. The experimental details of immunization are described in the Examples of the present application.

Preferably, such a method further comprises: producing hybridomas from the immunized animal host, wherein said hybridomas produce monoclonal antibodies; selecting the hybridoma (monoclonal) antibodies; and purifying and identifying the hybridoma (monoclonal) antibodies.

In one embodiment of the present invention, after the immunization, the animal may be sacrificed and the lymphocytes of said animal may be elicited. The lymphocytes can produce or be capable of producing antibodies that specifically bind to the protein used for immunization. Lymphocytes then are fused with myeloma cells using suitable fusing agents to form hybridomas cells. Examples of myeloma cell lines include, but are not limited to NS0. The hybridomas cells may be seeded and grow in suitable culture medium in 96- well culture plate with a density of one hybridoma cell per well. More preferably, nucleic acid encoding an inhibitor of apoptosis may be delivered into the myeloma cells to prevent the B-cell death induced by the production of auto-antigens. Said nucleic acids include, but are not limited to, anti-apoptosis genes, such as BCL-2. The experimental details of creating hybridomas cells are described in the Examples of the present invention.

Preferably, the anti-tumor agent may be identified by selecting hybridoma antibodies based on their differential binding reactivity to the type of cancer cells of interest. Hybridoma antibodies that bind to the type of cancer cells but not normal cells may be selected for further study. More preferably, the selected hybridomas can be further screened against multiple other types of cancer cells so that the antigen expression profiles of hybridoma antibodies can be established. As a result, the selected hybridomas may be categorized into two groups: one group of hybridoma antibodies may show that

their antigens are only expressed in a limited number of types of cancer cells. Accordingly, their antibodies can be employed for the treatment of a particular type of cancer. The other group of hybridoma antibodies may show that their antigens are expressed in multiple cancer cells. As a result, their antibodies may be used more broadly for the treatment of cancer. More experimental details are disclosed in the Examples of the present application.

Alternatively, the anti-tumor agent may also be selected by functional analysis. Apoptosis analysis may be performed to identify the hybridoma antibodies that are capable of inducing the cell-death of tumor cells. Thus, hybridomas inducing tumor death can be directly selected without the binding reactivity assay. Alternatively, the hybridomas can be screened based on the binding reactivity and then selected based on the death-inducing activity for tumor cells. In addition, the anti-tumor agents can be selected based on its ability to inhibit tumor cell proliferation.

Preferably, the method of identifying anti-tumor agents by lipid raft immunization comprises purifying and identifying the hybridoma antibodies. In other words, the method comprises purifying and identifying the antibodies produced by the hybridomas and the antigens that bind to the antibody. The molecular weight of the antigens can be determined by immunoprecipitation experiments. The antigens and antibodies of the selected hybridomas can be further purified by affinity chromatography and the antigen identified by microsequencing or by mass spectrometry. The experimental procedures of immunoprecipitation, affinity chromatography, and microsequencing can be found in the Examples of the present application. In addition, the anti-tumor agents can be selected based on the ability to inhibit tumor cell proliferation.

The antibody produced by hybridomas can be directly used as an anti-tumor agent. The anti-tumor activity of the antibodies produced by hybridomas can be verified by cell proliferation assay, xenograft model, and cell adhesion and migration assay. The experimental details are described in the Examples of the present application.

The method of identifying anti-tumor targets by lipid raft immunization comprises identifying the antigens that bind to the antibodies produced by hybridomas. The identity of the antigen can lead to the discovery of a group of potential anti-tumor agents. The examples for those anti-tumor agents include, but are not limited to, a molecule inhibiting the activities of said protein, a molecule down-regulating the expression of said protein, the molecule down-regulating the transcription of DNA encoding said protein, or anti-sense nucleic acid sequence of partial or full nucleic acid sequence encoding said protein.

Preferably, the present invention provides for a method of identifying anti-tumor agents for the treatment of prostate cancer by lipid raft immunization. More preferably, the method comprises: immunizing an animal with lipid raft preparations from prostate cancer cells; generating hybridomas; selecting monoclonal antibodies that are prostate cancer-positive and normal prostate-negative. The experimental details are described in the Examples of the present application.

More preferably, the prostate cancer cells are the cells of androgen-dependent prostate cancer (LNCaP), or androgen-independent prostate cancer (DU 145).

Preferably, the present invention provides for a method of identifying anti-tumor agents for the treatment of leukemia or lymphoma. More preferably, the method comprises: immunizing an animal with lipid raft preparations from leukemia or lymphoma cells; generating hybridomas; selecting hybridoma antibodies that are leukemia or lymphoma cell-positive and T-cell-negative; screening the cell death-inducing activity of hybridoma antibodies; obtaining hybridomas whose antibodies show specific killing of tumor cells. The experimental details are described in the Examples of the present application.

Preferably, the present invention provides for a hybridoma produced by the method of identifying anti-tumor agents by lipid raft immunization.

More preferably, the present invention provides for an antibody generated by the hybridoma.

More preferably, the present invention provides for an antigen that binds to the antibody generated by the hybridoma.

IV. Tumor Targets and Anti-tumor Agents and the Use Thereof.

The present invention provides for the tumor targets or the anti-tumor agents identified by the method of identifying anti-tumor targets by lipid raft proteomics and lipid raft immunization.

The present invention provides an isolated lipid raft derived form any cell, preferably from a tumor cell, more preferably from a prostate cancer (tumor) cell, or a leukemia or lymphoma cell. Preferably said isolated lipid raft is clustered with other lipid rafts derived from said prostate cancer cell. More preferably, said isolated lipid raft is a detergent resistant membrane (DRM).

Said isolated lipid raft derived from a prostate tumor cell may comprise a polypeptide that is differentially expressed in a prostate tumor cell. Preferably, said

polypeptide is selected from the group consisting of PMSA, CD10, Trop-1, ATP synthase, NCAM2, and CD222.

The present invention provides a monoclonal antibody that binds to an isolated lipid rafts, preferably an isolated lipid raft derived from a tumor cell, more preferably, said isolated lipid raft comprises a polypeptide that is differentially expressed in a type of tumor cell Preferably, said monoclonal antibody is a isolated monoclonal antibody.

Typically, the monoclonal antibody binds to both isolated lipid raft and the polypeptide that is a component of the isolated lipid raft and differentially expressed in the tumor cell where the lipid raft is derived from. Preferably, the monoclonal antibody binds to an exposed epitope of the polypeptide. The term "exposed epitope" refers to an epitope of said polypeptide that is on the surface of the lipid raft comprising said polypeptide, and not concealed due to the association of the polypeptide with the lipid raft. Thus, said antibody binds both to the lipid raft and said polypeptide. Preferably, said polypeptide is differentially expressed in prostate tumor cells, and is more preferably selected from the group consisting of PMSA, CD10, Trop-1, ATP synthase, NCAM2, and CD222. Accordingly, in addition to binding to said isolated lipid raft, said monoclonal antibody binds to or neutralizes PMSA, CD10, Trop-1, ATP synthase, NCAM2, or CD222.

More preferably, said anti-CD10 antibody specifically binds to prostate cancer cells but not binds to other types of cancer cells. Said anti-Trop-1 antibody reduces the colony formation of prostate cancer cells by more than 40%, 50%, 60%, preferably by about 65%.

The present invention provides an isolated lipid raft derived from a leukemia cell, wherein said isolated lipid raft comprising a polypeptide that is differentially expressed in said leukemia cell compared to a normal T cell. Preferably, said leukemia cell is a KG-1 cell.

The present invention provides a monoclonal antibody that binds to the isolated lipid raft derived from leukemia cells as well as the polypeptide that is differentially expressed in the leukemia cells. Preferably, said antibody induces apoptosis of the leukemia cell, preferably, by more than 50%, 60%, 70%, or 80%. More preferably, said polypeptide is HLA-DR antigen. The monoclonal antibody is preferably an isolated monoclonal antibody. In one aspect of the invention, the antibody comprises a heavy chain variable region of SEQ ID NO 1 and a light chain variable region of SEQ ID NO 2.

The present invention provides a method of treating prostate cancer comprising administering into a subject in need of such a treating a pharmaceutically effective amount of the identified anti-prostate tumor antibodies described herein.

The present invention also provides a pharmaceutical composition comprising a pharmaceutical carrier and the antibodies described herein.

Preferably, the anti-tumor agents, which are identified by using the methods in the present invention, may be employed for the treatment of disorders including, but not limited to, Hodgkin's Disease, Non-Hodgkin's Lymphoma, multiple myeloma, neuroblastoma, breast cancer, ovarian cancer, lung cancer, rhabdomyosarcoma, primary thrombocytosis, primary macroglobulinemia, small-cell lung tumors, primary brain tumors, stomach cancer, colon cancer, malignant pancreatic insulanoma, malignant carcinoid, urinary bladder cancer, premalignant skin lesions, testicular cancer, lymphomas, thyroid cancer, neuroblastoma, esophageal cancer, genitourinary tract cancer, malignant hypercalcemia, cervical cancer, endometrial cancer, adrenal cortical cancer, and leukemia, and other like cancers.

In particular, the identified anti-prostate tumor agents can be used for the treatment of prostate cancer. Said anti-prostate tumor agents include the antibodies isolated by lipid raft immunization of prostate cancer cells, such as the disclosed antibodies that bind to both the isolated lipid rafts and the identified prostate tumor targets including, but is not limited to, antibodies against PMSA, CD10, Trop-1, ATP synthase, NCAM2, or CD222.

The identified anti-leukemia or lymphoma tumor antigens can be used for the treatment of leukemia or lymphoma. Said anti-tumor agents include the antibodies isolated by lipid raft immunization of leukemia cells, such as the disclosed antibodies that bind to both the isolated lipid rafts and the identified leukemia target, such as HLA-DR.

Preferably, pharmaceutical compositions of the present invention are useful for parenteral administration, i.e., subcutaneously, intramuscularly and particularly, intravenously. The compositions for parenteral administration commonly comprise a solution of the antibody or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate,

sodium chloride, potassium chloride, calcium chloride, sodium lactate, histidine and arginine. The concentration of the antibodies in these formulations can vary widely, i.e., from less than about 0.01%, usually at least about 0.1% to as much as 5% by weight and are selected primarily based on fluid volumes, and solubilities in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for injection could be made up to contain 1 ml sterile buffered water, and 1-100 mg of an antibody. A typical composition for intravenous infusion can be made up to contain 250 ml of sterile Ringer's solution, and 10 mg of the inhibitor. Actual methods for preparing parentally administerable compositions are known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science (15th Ed., Mack Publishing Company, Easton, Pa., 1980), which is incorporated herein by reference.

The antibodies of this invention can be frozen or lyophilized for storage and reconstituted in a suitable carrier prior to use depending on the physical characteristics of the inhibitors. This technique has been shown to be effective with conventional antibodies and art-known lyophilization and reconstitution techniques can be employed.

For the purpose of treatment of disease, the appropriate dosage of antibodies will depend on the severity and course of disease, the patient's clinical history and response, the toxicity of the inhibitors, and the discretion of the attending physician. The inhibitors are suitably administered to the patient at one time or over a series of treatments. The initial candidate dosage may be administered to a patient. The proper dosage and treatment regime can be established by monitoring the progress of therapy using conventional techniques known to the people skilled of the art.

The amount of active ingredients that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors, including the activity of the specific inhibitor employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy, and can be determined by those skilled in the art.

The compositions can be administered for prophylactic and/or therapeutic treatments, comprising preventing, inhibiting, and reversing cancer cell proliferation, or inducing the apoptosis of the cancer cells. An amount adequate to accomplish the desired

effect without toxic effect is defined as a "pharmaceutically effective amount" and will generally range from about 0.01 to about 100 mg of antibody per dose. Single or multiple administrations can be carried out to achieve the desired therapeutic effect.

Antibodies disclosed herein are useful in diagnostic and prognostic evaluation of diseases and disorders, particularly cancers associated with the tumor target expression. A t each stage of disease, monoclonal antibodies against the identified tumor target may be used to improve diagnostic accuracy and facilitate treatment decisions. Labeled monoclonal antibodies can detect abnormal cells at an early stage, because of their expression of tumor antigens. Once cancer is diagnosed, accurate staging is important in deciding on the most appropriate therapy. Later, during follow-up of surgery, rising serum levels of tumor antigens may indicate recurrence before it can be detected by conventional methods.

Methods of diagnosis can be performed in vitro using a cellular sample (e.g., blood sample, lymph node biopsy or tissue) from a patient or can be performed by in vivo imaging.

Compositions comprising the antibodies of the present invention can be used to detect the presence of a tumor target in a type of cancer cells, for example, by radioimmunoassay, ELISA, FACS, etc. One or more labeling moieties can be attached to the humanized immunoglobulin. Exemplary labeling moieties include radiopaque dyes, radiocontrast agents, fluorescent molecules, spin-labeled molecules, enzymes, or other labeling moieties of diagnostic value, particularly in radiologic or magnetic resonance imaging techniques.

Examples

Example 1

This example describes the identification of anti-tumor targets for the treatment of prostate cancer by using lipid raft proteomics.

In the present invention, molecules critical to the treatment of prostate cancer were sought by initially detecting differential expression of proteins in normal cells and the prostate tumor cell lines. Lipid rafts of each cell line were isolated and studied subsequently.

MATERIALS AND METHODS

a. Lipid Raft Preparation

Lipid rafts were prepared as described in Green et al, *J. Cell Biol.* 146, 673-682 (1999). Briefly, cells (8.0 X 10^6 cells / sample) were lysed in 0.1% vol/vol Brij-58, 20 mM Tris HCl, pH 8.2, 140 mM NaCl, 2 mM EDTA, 25 μ g/ml aprotinin, 25 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride for 10 minutes on ice. Cells were homogenized using 10 strokes of a Dounce homogenizer, then lysed 20 minutes more on ice. The resulting lysate was adjusted to 40% wt/wt sucrose and applied onto a 60% wt/wt sucrose cushion. A sucrose step-gradient consisting of 25% wt/wt sucrose and 5% wt/wt sucrose were layered on top of the lysate. Gradients were centrifuged 18 hours at 170,000 x *g* at 4 °C in a SW55 rotor. Fractions (0.2 ml) were taken from the top of the gradient. Lipid rafts float to the interface of the 25% and 5% sucrose layers (Fractions 7 and 8). The amount of protein in each fraction was determined using the BCA Protein Assay Kit. Protein was concentrated by centrifugation at 2000 x *g* in Vivaspin 6 PES membrane columns (molecular weight cut off = 10,000 kDa).

b. Electrophoresis and Western Blotting

Lipid raft proteins were separated by SDS-PAGE on a 4-20% gradient gel and then electrotransferred onto a polyvinylidene difluoride membrane (PVDF). The membrane was blocked for 1.5 hours at room temperature in PBS with 5% milk. The membrane was then incubated with 0.4 μ g/ml mouse anti-ATP synthase (Molecular Probes, catalog # A-11144) in PBS with 1% BSA and 0.5% Tween-20 for 2 hours at room temperature. After extensive washing, the membrane was incubated with HRP-conjugated goat antibodies specific for mouse IgG for 1 hour at room temperature in PBS with 1% BSA and 0.5% Tween-20. After extensive washing, blot was developed using enhanced chemiluminescence followed by fluorography.

c. N-terminal Sequencing

Proteins to be sequenced were separated by SDS-PAGE on a 4-20% gradient gel and then electrotransferred onto a PVDF membrane. The membrane was stained for 2 minutes using colloidal Coomassie and then destained in water. The resulting bands were excised and subjected to N-terminal Edman sequencing as described by Miller, *Methods: A Companion to Methods in Enzymology* 6, 315 (1994). Results were confirmed using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) peptide-mass profiling.

d. MALDI-TOF peptide-mass profiling

Proteins to be analyzed were separated by SDS-PAGE on a 4-20% gradient gel. Alternatively, 2-dimensional electrophoresis was used to further separate lipid raft proteins using an IPGphor Isoelectric Focusing System according to the manufacturers protocol (Amersham Pharmacia Biotech, Piscataway, NJ). Proteins were visualized by staining the resulting gel with 0.05% Coomassie Blue R250, 50% methanol, 10% acetic acid in water followed by destaining in 15% methanol, 10% acetic acid in water.

Protein band or spot of interest was excised with a razor blade and equilibrated in 100 mM Tris HCl, pH 8.5 at room temperature for 45 minutes. The solution was replaced with 150 µL of 2 mM DTT in 100 mM Tris-HCl, pH 8.5. The samples were incubated with agitation for 30 minutes at 60 °C. The solution was replaced with 150 µL of 20 mM iodoacetic acid in 100 mM Tris-HCl, pH 8.5. The samples were incubated in the dark at 37°C for 30 minutes. The solution was replaced with 150 µL of equal parts 100 mM Tris-HCl, pH 8.5 and acetonitrile. The tubes were shaken vigorously at 37 °C for 45 minutes. This step was repeated until the gel bands were clear. The solution was removed and the gel slices were dried in a SpeedVac on low vacuum strength for 15 minutes. The gel bands were re-swelled with 0.25-0.5 µg of a concentrated endo-protease Lysine-C or trypsin solution, then covered with 50-80 µL of 100 mM Tris-HCl, pH 8.5, 10% acetonitrile and incubated with agitation for 18 hours at 37 °C. After digestion the samples were stored at 4-8°C.

The digest solution was removed from the micro-centrifuge tube, acidified with 10% trifluoroacetic acid (TFA) in water v/v to 1% TFA v/v, desalted and concentrated using a C18 Zip-Tip. The micro-column eluate was combined 1:1 with 10 mg/ml alpla-Cyano-4-hydroxycinamic acid in 60% acetonitrile and spotted on a MALDI-TOF sample plate. A close external calibrant with the approximate concentration of the sample was spotted adjacent to the sample position on the MALDI plate. The calibrant was prepared by diluting a pre-made calibration mix consisting of angiotensin II fragment 1-7 and adrenocorticotropic hormone fragment 18-39 with Zip-Tip eluant. The diluted calibration mixture was combined 1:1 with matrix solution. The sample and calibrant spots were dried simultaneously at room temperature.

MALDI mass fingerprints were acquired on a Perceptive Biosystems Voyager DE Pro MALDI-TOF in reflector mode with delayed extraction and positive polarity. Approximately 100-300 shots from a 20 KV laser were accumulated. During spectrum acquisition a resolution calculator was employed to ensure accurate calibration. After the spectra were acquired and calibrated, the monoisotopic masses were automatically

selected using the de-isotoping function on Voyager Software. The calibrated monoisotopic peak lists were exported into ProteinProspector MS-Fit version 3.2.1 and searched against the largest non-redundant database available from the National Center for Biotechnology Information (NCBInr).

e. Flow cytometry staining

Flow cytometry was used to screen hybridoma supernatants for the presence of cell surface binding antibodies. The cells (2×10^5) were resuspended in 100 µL ice cold PBS with 10 µL tissue culture supernatant on ice for 1 hour. After extensive washing, cells were incubated with phycoerythrin-conjugated goat antibodies specific for mouse IgG for 30 minutes on ice. Cells were washed again and cell surface bound antibody was detected using a Becton Dickenson FACScan. Additionally, hybridoma supernatants were similarly screened on many cancer cell lines or whole blood to test for specificity.

f. Immunofluorescence

LNCaP cells were grown on glass coverslips undisturbed for two days. Cells were fixed with 3% paraformaldehyde in PBS for 15 minutes. After being washed with PBS, cells were incubated in 50 mM NH_4Cl in PBS for 10 minutes. After washing with PBS, cells were subsequently incubated with 5% goat serum in PBS for 30 minutes followed by staining with 5 µg/ml anti-ATP synthase or anti-Trop-1 (EpCAM) in 2.5% goat serum in PBS for 1 hour at room temperature. Cells were stained with anti-MHC class II (Kostelny et al *Int. J. Cancer* 93, 556 (2001)) as a negative control. After washing with PBS, bound antibody was detected by incubating cells with Alexa 488-conjugated goat antibodies specific for mouse IgG in 2.5% goat serum in PBS for 30 minutes at room temperature. After extensive washing, glass coverslips were mounted in a solution containing Mowiol 4-88, glycerol, and 150 mM Tris HCl, pH 8.5. Slides were placed at 4 °C overnight before viewing. Cells were analyzed by fluorescence microscopy on a Nikon Optiphot 2 microscope.

g. LNCaP Proliferation

LNCaP cells were plated at 20,000 cells/well into a 96 well tissue culture plate. After cells were allowed to grow undisturbed for two days, antibodies (5 µ g/ml anti-ATP synthase, anti-Trop-1 (EpCAM), or anti-MHC class II) were added and incubated with the cells for 24 hours. Cell proliferation was measured using the AlamarBlue vital dye indicator assay. AlamarBlue reagent was added to each well and the plates were incubated for 3 to 4 hours at 37 °C to allow for fluorescence development. Fluorescence

was detected at λex=530nm, λem=590 nm. Data are expressed as the mean +/- SEM of 4 replicates.

h. Soft Agar Colony Formation Assay

For anchorage-independent cell growth, a soft agar colony formation assay was performed in a six-well plate. Each well contained 2 mL of 1% agar in complete medium as the bottom layer. The top layer contained 2 mL 0.5% agar in complete medium, 1000-10000 LNCaP cells, and 5 μg/mL mAb (anti-ATP synthase, or anti-Trop-1). One mL complete medium was added and the cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere for up to 20 days. One mL complete medium was added once a week. Media was removed and the colonies were stained with 0.005% crystal violet in PBS for 2 hours. The number of colonies was determined by counting them under an inverted phase-contrast microscope at 100X, and a group of 10 or more cells were counted as a colony.

RESULTS AND DISCUSSION

Differential expression of ATP synthase in prostate tumor cells

Lipid rafts were extracted from normal prostate cells and three widely used prostate cancer cells – LNCaP, DU145, and PC-3. The protein expression of each sample was compared using one-dimensional electrophoresis together with silver staining. Several protein bands in the one-dimensional electrophoresis gel appeared only in the prostate cancer cell lines, indicating that they are candidate proteins related to the prostate cancer. One of the candidate proteins with molecular weight of approximately 50kD (denoted by an arrow with a "*" in Figure 1) was selected for N-terminal sequencing. The sequencing result indicated that it was the β-subunit of ATP synthase.

The differential protein expression was also confirmed by two-dimensional electrophoresis and then visualized by silver staining. Protein spots that are present in the LNCaP sample, but not the normal prostate sample are denoted with arrows (16 spots identified), as shown in Figure 2. Many of these lipid raft proteins were excised and subjected to MALDI-TOF peptide mass profiling analysis. The identities of 5 of them are shown in Table 1. The locations of these proteins on the 2-D gel are labeled with numbers as shown in Figure 2. We found 2 subunits of ATP synthase (α and β subunits), 2 voltage-dependent anion channel/porin proteins, adenine nucleotide translocator, and prohibitin. All of these 5 identified proteins are mitochondria proteins but somehow are

associated with lipid rafts of LNCaP cells. It is not known how many of these lipid raft-associated proteins are exposed to the outer surface of the cell membrane and therefore are accessible to antibodies. As several anti-ATP synthase antibodies are commercially available, we used one of them to confirm that some ATP synthase molecules are located in lipid rafts and they are accessible to antibodies.

Western blot analysis of lipid rafts using anti-α-subunit ATP synthase antibody further confirmed the correlation between the ATP synthase and prostate cancer. As shown in Figure 3, positive staining appeared in all three prostate cancer cell lines, but not the normal cells, indicating that ATP synthase was present in lipid rafts of prostate cancer cells but not normal cells. In addition, similar Western blot analysis of lipid rafts from cancer cells of different origins indicates that some cancer cell lines, such as the AML cell lines KG-1 and THP-1, the breast cancer cell line MCF-7, the colon cancer cell line LS180, and the bladder cancer cell line RT4 also express ATP synthase in lipid rafts (Figure 4).

Cell surface localization of ATP synthase

To investigate the surface localization of the ATP synthase, prostate tumor cell line LNCaP was analyzed by FACS staining and immunofluorescence microscopy. As shown in Figure5, FACS staining by anti-ATP synthase antibody in LNCaP tumor cells revealed about 25.9% of cells had ATP synthase cell surface expression, Staining with an antibody against Trop-1 (EpCAM), a cell surface protein expressed on cancer cells, showed surface localization in 92.8% of the cells. Similar analysis of the AML cell line THP-1 by flow cytometry also showed that these cells have ATP synthase on their cell surface (Figure 6). ATP synthase was also showed to be expressed on the surface of the androgen-independent cell line DU 145 by a similar analysis.

The surface localization of ATP synthase was further evidenced by the immunofluorescence microscopy. As shown in Figure 7, immunostaining of LNCaP cells with anti-ATP synthase gave rise to positive cell surface staining. The colocalization of Trop-1 (EpCAM) and ATP synthase indicated that ATP synthase was indeed expressed on the surface of prostate tumor cells.

Moreover, FACS staining of LNCaP tumor cells growing in Matrigel® demonstrated a much higher percentage of cells exhibiting a positive surface staining of ATP synthase, confirming that ATP synthase cell surface expression can be modulated by cellular environment, and this may play important roles in the real biological environment (data not shown).

Inhibition of prostate cancer cell proliferation in the presence of anti-ATP synthase

As shown in Figure 8, LNCaP tumor cell proliferation can be reduced by antibodies specific for ATP synthase. This reduction in cellular proliferation ranges from 12.5% to 27.8%. This substantial inhibition of cell proliferation suggests a potential of anti-ATP synthase antibody as an anti-tumor agent for treating prostate cancer.

The above experiments demonstrate that ATP synthase is closely related to the prostate cancer cells. It is localized on the surface of prostate tumor cells, and may play a key role in the biological activities of prostate cancer cells. Blocking the activity of ATP synthase by antibodies inhibits the prostate tumor cell growth, suggesting the possibility of clinical application of ATP synthase inhibitors as anti-tumor agents for treating prostate cancer.

Inhibition of prostate cancer cell colony formation in soft agar in the presence of anti- ATP synthase

Transformed cancer cells are resistant to anchorage-independent growth inhibition and are able to growth in soft agar without attaching to cell matrix. Formation of colonies (three-dimensional growth under tissue culture growth conditions) of cancer cells in soft agar is often correlated to the aggressiveness of the tumor in vivo. To assess whether anti-ATP synthase has any anti-cancer activity, we used it inhibit LNCaP colony formation in vitro. As shown in Figure 9, LNCaP colony formation can be reduced by an antibody specific for ATP synthase (anti-α subunit) at 5 μg/ml. This reduction in colony formation by anti-ATP synthase could be as high as 95%, whereas the action of an anti-Trop-1 antibody (Ep-CAM) antibody was less impressive, at about 68%. This substantial inhibition of colony formation suggests a potential of anti- ATP synthase antibody as an anti-tumor agent for treating prostate cancer.

AML cell death induction in the presence of anti-ATP synthase

The anti-cancer activity of anti-ATP synthase can also be demonstrated in the AML cell line THP-1, which expresses ATP synthase on the cell surface. Incubation of THP-1 cells with anti-ATP synthase at 5 μg/ml for 24 hours led to substantial cell death as assayed by flow cytometry (Figure 10). Compared to PBS or an irrelevant antibody control, the specific killing induced by anti-ATP synthase was 25%. This anti-AML activity suggests a potential of antiATP synthase antibody as anti-leukemia or lymphoma agent to treat hematological malignancies.

The above experiments demonstrate that ATP synthase expression is closely related to certain cancer cells. It is localized on the surface of prostate and AML cancer cells, and may play a key role in the biological activities of prostate and AML cancer cells. Blocking the activity of ATP synthase by antibodies inhibits the prostate cancer cell growth and colony formation, and induces cell death in AML cancer cells, suggesting the possibility of clinical application of ATP synthase inhibitors as anti-tumor agents for treating prostate cancer and AML.

Example 2

This example describes the identification of anti-tumor targets in various cancer cells by using lipid raft proteomics.

Lipid raft protein expressions were compared among various cancer cell lines. Lipid rafts were extracted from normal trophoblast cells (BeWo and JEG-3) and various cancer cells, including colon cancer cells (HT-29, LS180, and Colo205), breast cancer cells (MCF-7), prostate cancer cells (LNCaP), pancreas cancer cells (PANC-1), and lung cancer cells (NCI-H292). The protein expression of each sample was compared using one-dimensional electrophoresis together with silver staining. As shown in Figure 11, many protein bands were shared among the various cell lines, while many proteins are differentially expressed. In addition, there are approximately 120 protein bands per lane, confirming that lipid rafts are not too complex to be studied. The differential protein expressions were also confirmed by two-dimensional electrophoresis and Western blot analysis as described in Example 1. The protein bands differentially expressed in cancer cells were isolated, sequenced, and identified as candidates for tumor related molecules as described in Example 1.

Example 3

This example describes identifying the anti-prostate tumor agent by the immunization of lipid rafts from LNCaP cell lines.

MATERIALS AND METHODS

a. Lipid Raft Preparation

See Example 1.

b. Lipid Raft Immunization

Lipid raft proteins (approximately 5 μg) were mixed together with 50 μL Ribi®, and then injected into the foot pad of a BALB/c mouse. Mice were boosted with 50 μ L

of lipid raft proteins in Ribi® on day 7 and day 14. Three days after the last boost, the mice were sacrificed and the hind leg lymph node was harvested. The lymph node was washed in pre-warmed DMEM and then ground using a Dounce homogenizer. After 5 gentle strokes, the cell suspension was removed into the plastic tube. This process was repeated four more times, each time adding 5 ml of fresh DMEM. The lymphocytes were pooled and washed 3 times in DMEM. The lymphocytes were mixed with the appropriate number of pre-washed fusion partner NS0/BCL-2 (NS0 transfectant expressing the mouse BCL-2 cDNA) to yield ratio of 2-3 lymphocytes for every 1 NS0. The mixture was pelleted and warmed at 37°C for 1 min. Pre-warmed 50% PEG was slowly added onto the pellet and then cells were centrifuged at 300 X *g* for 3 minutes at room temperature. Five mL DMEM was added and then 10 mL DMEM with 10%FBS and 1%P/S was added. The cells were then centrifuged 5 minutes at 300 x *g* at room temperature. The pellet was resuspended in HAT selection medium (DMEM with 20% fetal bovine serum, 2 mM glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.1 mM sodium hypoxanthine, 16 μM thymidine, 20 μM aminopterin, 2X Origen cloning factor, 10 mM HEPES, 50 μM beta-mercaptoethanol, 0.2 units/mL penicillin, 0.2 μg/mL streptomycin) to yield 0.25 X 10^6 lymphocytes/mL. Cells were aliquoted into ten 96-well flat bottom plates at 200 μL per well for the selection of hybridomas.

c. Flow cytometry screening

Flow cytometry was used to screen hybridoma supernatants for the presence of cell surface binding antibodies. The cells (2 X 10^5) were resuspended in 100 μL ice cold PBS with 10 μL tissue culture supernatant on ice for 1 hour. After extensive washing, cells were incubated with phycoerythrin-conjugated goat antibodies specific for mouse IgG for 30 minutes on ice. Cells were washed again and cell surface bound antibody was detected using a Becton Dickenson FACScan. Additionally, hybridoma supernatants were similarly screened on many cancer cell lines or whole blood to test for specificity.

d. Affinity Purification of Antigen

Cells (5 X 10^8) were lysed in 1% vol/vol NP-40, 0.5% wt/vol deoxycholate, 20 mM Tris HCl, pH 8.2, 150 mM NaCl, 1 mM EDTA, 25 µg/ml aprotinin, 25 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride for 1 hour on ice with frequent mixing. Lysate was centrifuged for 20 minutes at 300 x *g* to remove nuclei and debris. Antigens were purified by standard hybridoma affinity chromatography techniques as described in Hill et al, *J. Immunol.* 152, 2890-2898 (1994).

e. Antigen Grouping

Cells (2 X 10^7) were cell surface iodinated as described (Landolfi and Cook, *Mol. Immunol.* 23, 297-309 (1986)). Cells were then lysed in 1% NP-40, 0.5% deoxycholate, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF for 1 hour on ice. Cell lysate was centrifuged at 14,000 x *g* for 5 minutes to remove nuclei and debris. Cell lysate was pre-cleared with rotation by incubation with Gamma Bind Plus Sepharose beads for 2 hours at 4 °C. The beads were spun down and the cell lysate was then aliquoted into Eppendorf tubes containing Gamma Bind Plus Sepharose beads that had been pre-incubated with hybridoma supernatant. The tubes were rotated overnight at 4 °C. After extensive washing, bound antigen was eluted from the beads by boiling in the presence of 5% wt/vol SDS, 125 mM Tris-HCl, pH 6.8, and 4% vol/vol β-mercaptoethanol, and 50% vol/vol glycerol. Proteins were then subjected to SDS-PAGE. After electrophoresis, the gel was fixed for 30 minutes with 60% H_2O/30% methanol/10% acetic acid. The gel was then washed for 30 minutes with water then dried down. The dried gel was put on film (Kodak® Biomax MS® film with appropriate Biomax MS® screen) overnight

f. Soft Agar Colony Formation Assay

For anchorage-independent cell growth, a soft agar colony formation assay was performed in a six-well plate. Each well contained 2 mL of 1% agar in complete medium as the bottom layer. The top layer contained 2 mL 0.5% agar in complete medium, 1000-10000 LNCaP cells, and 5 µg/mL mAb (anti-ATP synthase, anti- NCAM2, or anti-Trop-1). One mL complete medium was added and the cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere for up to 20 days. One mL complete medium was added once a week. Media was removed and the colonies were stained with 0.005% crystal violet in PBS for 2 hours. The number of colonies was determined by counting them

under an inverted phase-contrast microscope at 100X, and a group of 10 or more cells were counted as a colony.

g. LNCaP Proliferation

LNCaP cells were plated at 20,000 cells/well into a 96 well tissue culture plate. After cells were allowed to grow undisturbed for two days, antibodies (5 μg/ml anti-ATP synthase, anti-NCAM2, anti-Trop-1, or anti-MHC class II) were added and incubated with the cells for 24 hours. Cell proliferation was measured using the AlamarBlue vital dye indicator assay. AlamarBlue reagent was added to each well and the plates were incubated for 3 to 4 hours at 37 °C to allow for fluorescence development. Fluorescence was detected at λex=530nm, λem=590 nm. Data is expressed as the mean +/- SEM of 4 replicates.

RESULTS AND DISCUSSIONS

One BALB/c mouse was immunized with a lipid raft preparation from the prostate cancer cell line LNCaP. After two boosts, lymphocytes were isolated from the mouse lymph nodes and fused with myeloma NS0 cells to generate hybridomas. A total of about 700 hybridomas were generated and supernatant from each hybridoma was screened by flow cytometry for binding to LNCaP. About 203 supernatants tested positive, and they were further tested for binding to normal prostate cells. Thirty-four of these supernatants tested negative for binding to normal prostate cells. Antibodies from these 34 hybridomas were further tested against multiple cancer lines by flow cytometry to determine whether they are LNCaP specific, prostate cancer specific, or pan-cancer specific. The cancer cell lines we used include: DU 145 (prostatic), PC-3 (prostatic), PANC-1 (pancreatic), RT4 (bladder), HT-29 (colorectal), NCI-H292 (lung), T-47D (breast), and NIH:OVCAR-3 (ovarian). In addition, a primary, non-transformed HUVEC line (human umbilical vein endothelial cells) was used to ensure that these antibodies do not cross-react with normal endothelial cells. The 34 LNCaP reacting hybridomas can be divided into two main groups based on their antigen expression profiles: antibodies from 20 of these hybridomas (Table 2) showed that their antigens are expressed only in a limited number (1-3) of cancer cell lines and 14 (Table 3) showed that their antigens are expressed in multiple lines. A flow chart describing how these tumor-associated, LNCaP lipid raft-derived antigens were identified by the hybridoma technology is shown in Figure 12.

We performed an immunoprecipitation experiment to determine the molecular weight of the antigens that showed limited expression profiles. Of the twenty antibodies used, three predominant antigens of MW 98 (recognized by 3 antibodies), 100 (recognized by 6 antibodies), and 120 kD (recognized by 4 antibodies) were identified (Figure 13, see also the last column of Table 2). Seven antibodies were not able to immunoprecipitate antigen for molecular weight determination. The antigen grouping results indicate the hybridomas of Table 1 covered a minimum of four to a maximum of ten antigens. Similarly antigen grouping by immunoprecipitation may also apply to those antigens showing broad expression profiles (antigens as defined by hybridomas of Table 3) to reduce the number of tumor-associated antigens that need to be characterized.

Lipid raft tumor-associated antigen NCAM2

The identity of the antigen defined by the hybridoma P3-53 had been determined. The monoclonal antibody produced by hybridoma P3-53 (Table 2) immunoprecipitated a major protein band of 120 kD and a minor band of 110 kD (see Figure 13, Lane 3). The P3-53 antigen is expressed only in the prostate cell line LNCaP and breast cell line T-47D. The monoclonal antibody from P3-53 was conjugated to CNBr-activated Sepharose to generate an affinity column (20 mg conjugated to a 2 ml column). LNCaP whole cell lysate was prepared from 2 $x10^8$ cells as described in MATERIAL AND METHODS and passed onto the P3-53 affinity column. After extensive washing, the retained protein was eluted with low pH buffer. About 5 μg of the P3-53 antigen was purified. SDS-PAGE analysis followed by silver staining revealed the P3-53 antigen consisted of a major protein band of 120 kD and a minor band of 110 kD. The purified antigen was subjected to microsequencing analysis and the result showed that it had a NH_2-terminal sequence of X-L-QV-T-I-S-L-S-K, where X was probably "L", but might also be "G". This sequence was searched against the entire NCBInr database using Protein Prospector. Only one human protein with the NH_2-terminal sequence of L-L-Q-V-T-I-S-L-S-K matched the determined P3-53 antigen sequence. The human protein is called neural cell adhesion molecule 2 (NCAM2, NCBI protein accession number 4758764, see also Paoloni-Giacobino et al, *Genomics* 43, 43-51 (1997)), which is a homologue of a murine protein called Rb-8 neural adhesion molecule (RNCAM, NCBI protein accession number 3334269, see also Alenius,M. and Bohm, S., *J. Biol. Chem.* 272, 26083-26083 (1997) and Yoshihara et al. *J. NeuroSci.* 17: 5830-5842 (1997). The identification of the antigen recognized by P3-53 was confirmed to be NCAM2 by MALDI-TOF peptide-mass profiling as described in the MATERIALS AND METHODS in Example 1.

The sequence of RNCAM predicted molecules having an extracellular region of 5 immunoglobulin C2-type domains followed by two fibronectin type III domains. Alternative splicing of the NCAM2 and RNCAM transcripts generate two isoforms: the long form containing a transmembrane domain and the short form containing a glycosylphosphatidylinositol-anchor attached to the membrane. The expression of RNCAM is restricted to the olfactory neurons in the brain and in the nasal vomeronasal organ. The transcript of RNCAM is not detectable in lung, gut, liver, heart, testis and kidney. The function of NCAM2 or RNCAM is not known, but the molecule may play a role in selective axon projection. NCAM2 was also shown to be a homophilic adhesion molecule (see Yoshihara et al. *J. NeuroSci.* 17: 5830-5842 (1997)). Thus we found that certain prostate and breast cancer cell lines express this protein marker that is neural in origin. Accordingly, because NCAM2 is expressed in a substantial percentage of prostate and breast cancer cells, an antibody to NCAM2 provides an effective treatment for prostate or breast cancer. Antibodies are better drugs than small molecules against cancer cells expressing neural markers because they do not cross the blood-brain barrier to potentially have toxic effects on normal neurons.

Inhibition of prostate cancer cell proliferation by anti-NCAM2 antibodies

As shown in Table 2 and Figure 13, P3-53, P9-64, P10-28, and P10-29 all immunoprecipitated an antigen with a molecular weight of 120/110. The P3-53 antigen was identified as NCAM2. We tested the anti-cancer activity of 4 anti-NCAM2 antibodies in a proliferation assay using the prostate cancer cell line LNCaP. The results are shown in Figure 14. One of the 4 antibodies, namely P9-64, had significant inhibition activity against proliferation of LNCaP cells. This reduction in cellular proliferation by P9-64 was about 39%. P10-28 and P10-29 had about 16 and 11% inhibition activity, respectively, and P3-53 was not effective. . This substantial inhibition of cell proliferation by some anti-NCAM2 antibodies suggests a potential of anti- NCAM2 antibody as an anti-tumor agent for treating prostate cancer.

Inhibition of prostate cancer cell colony formation by anti-NCAM2 antibodies

Transformed cancer cells are resistant to anchorage-independent growth inhibition and are able to growth in soft agar without attaching to cell matrix. Formation of colonies (three-dimensional growth under tissue culture growth conditions) of cancer cells in soft agar is often correlated to the aggressiveness of the tumor *in vivo*. To assess whether the four anti-NCAM2 antibodies any anti-cancer activity, we used them inhibit LNCaP colony formation in vitro. As shown in Figure 15, LNCaP cancer cell colony formation

can be reduced by some anti-NCAM2 antibodies at 5 μg/ml. As in the proliferation assay, the most potent inhibitor is P9-64. It inhibited colony formation by 42%. P10-28 and P10-29 inhibited about 22% and 36% respectively. The inhibitory activity of P3-53 was again low, at about 10% inhibition. The substantial inhibition of colony formation by some anti-NCAM2 antibodies suggests a potential of anti- NCAM2 antibody as an anti-tumor agent for treating prostate cancer.

The above experiments demonstrate that NCAM2 expression is closely linked to some types of prostate cancer cells. It is localized on the surface of cancer cells, and may play a key role in the biological activities of prostate cancer cells. Blocking the activity of NCAM2 by antibodies inhibits prostate cancer cell growth, suggesting the possibility of clinical application of NCAM2 inhibitors as anti-tumor agents for treating prostate cancer.

Lipid raft tumor -associated antigens PSMA and CD10

The identities of two more antigens defined by hybridomas listed in Table 2 have also been determined. The monoclonal antibody produced by hybridoma P12-22, representing 6 hybridomas (Table 2), immunoprecipitated a protein band of 100 kD (see Figure 13, lane 20). The P12-22 antigen is expressed in the prostate cancer cell line LNCaP. The monoclonal antibody from P12-22 was conjugated to CNBr-activated Sepharose to generate an affinity column (20 mg conjugated to a 2 ml column). LNCaP whole cell lysate was prepared from 2 $x10^8$ cells as described in MATERIAL AND METHODS and passed onto the P12-22 affinity column. After extensive washing, the retained protein was eluted with low pH buffer. About 15 μg of the P12-22 antigen was purified. SDS-PAGE analysis followed by silver staining revealed the P12-12 antigen had a molecular weight of 100 kD. The purified antigen was subjected to microsequencing analysis and the result showed No Edman degradation products were obtained, indicating the NH_2-terminus of this antigen might have been blocked. The purified P12-22 antigen was then subjected to MALDI-TOF peptide mass profiling as described in MATERIALS AND METHODS in Example 1. This method identified the P12-22 antigen as prostate specific-membrane antigen (PSMA) also known as folate hydrolase (NCBI accession number 20561181), a well-known prostate cancer associated antigen that is expressed in LNCaP, but not in PC-3 or DU 145. This antigen is a type II transmembrane protein that is highly expressed in prostate cancer. The NH_2-terminus of this protein is known to be blocked (see Israeli et al. Cancer Research 53, 227-230 (1993)).

The monoclonal antibody produced by hybridoma P10-82, representing 3 hybridomas (Table 2), immunoprecipitated a protein band of 98 kD (see Figure 13, lane 19), which is expressed mostly in the prostate cancer cell line LNCaP. The P10-82 antigen was purified by the antibody affinity column affinity described above. About 10 μg of the antigen was obtained. Microsequencing analysis of this protein yielded no Edman degradation products, but MALDI-TOF peptide mass profiling identified the P10-82 antigen as CD10, also known as neutral endopeptidase (NCBI accession number 14733461). This is also a type II transmembrane protein. Both PSMA and CD10 were previously identified as prostate cancer-associated antigens (see Gong et al. *Cancer Metastasis Rev.*18, 483-490 (1999) for review of PSMA, and Krongrad et al. *Urol. Res.* 25,113-116 (1997) and Sumitomo et al. *J. Clin. Invest.* 106, 1399-1407 (2000) for CD10), although they were not known to be lipid-raft associated. The finding of these known cancer-associated antigens by the lipid raft immunization approach provides a good validation of the method.

Lipid raft tumor-associated antigens Trop-1 (EpCAM) and CD222

We also performed an immunoprecipitation experiment to determine the molecular weight of the antigens that showed broad expression profiles (Table 3). The molecular weights of 8 antigens were determined and are summarized in Figure 16 and Table 3. Unlike the antigens in Table 2, the molecular weights of 8 antigens determined in Table 3 appeared to be different from each other. No immunoprecipitation result was obtained with the P8-20 antibody but the antigen was identified as Trop-1 (EpCAM), which we knew as a lipid raft associated tumor antigen (unpublished observation). The identity of P8-20 antigen as Trop-1 was determined by staining of Trop-1/mouse myeloma NS0 transfectant with all 14 antibodies listed in Table 2 by flow cytometry. Only P8-20 antibody stained positively with the Trop-1 transfectant.

By using affinity chromatography and MALDI-TOF, two more antigens in Table 3 were also identified. The P7-69 antigen was identified as PSMA, the tumor-associated antigen that we previously identified in Table 2. The P11-65 antigen (>220 kD) was identified as CD222, also known as mannose-6-phosphate receptor or insulin-like growth facto II receptor (NCBI accession number 13642251). It is a ubiquitously expressed multifunctional type I transmembrane protein. Its main functions include internalization of insulin-like growth factor II and internalization and sorting of lysosomal enzymes and other mannose-6-phophate-containing proteins (Korenfied, *Annu. Rev. Biochem.* 61, 307-330 (1992)). The majority of CD222 molecules (90-95%) are located intracellularly in

normal cells; only 5-10% is presented on the membrane surface. CD222 is also a receptor of TGF-β latency associated peptide (LAP), the angiogenesis-inducing protein proliferin, plasminogen, and retinoic acid. The data in Table 3 indicate the epitope on CD222 recognized by P11-65 antibody is highly expressed in many cancer cell lines but detectable only weakly in one of the three normal cell line tested.

Example 4

This example describes generation and characterization of anti-KG-1 lipid raft hybridomas.

MATERIALS AND METHODS

a. Flow cytometry screening

Flow cytometry was used to screen hybridoma supernatants for the presence of cell surface binding antibodies. The cells (2×10^5) were resuspended in 100 μL ice cold PBS with 10 μL tissue culture supernatant on ice for 1 hour. After extensive washing, cells were incubated with phycoerythrin-conjugated goat antibodies specific for mouse IgG for 30 minutes on ice. Cells were washed again and cell surface bound antibody was detected using a Becton Dickenson FACScan. Additionally, hybridoma supernatants were similarly screened on whole blood to test for specificity. Normal blood cell populations were purified as described from peripheral blood from volunteers (Brown *Methods in Cell Biol.* 45, 147 (1994)) or purchased from the Stanford Medical School Blood Center (Stanford, CA).

b. Apoptosis

Flow cytometry was used to screen hybridoma supernatants for the ability to induce apoptosis of acute myelogenous leukemia cancer cells. KG-1 cells or THP-1 cells (2×10^5) were resuspended in 100 μL media with 100 μL tissue culture supernatant for 24 hours at 37 °C. Cells were centrifuged and then incubated with FITC-conjugated annexin V and propidium iodide in 10 mM HEPES, pH 7.4, 150 mM NaCl, .5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ for 15 minutes at room temperature. After extensive washing, cells were analyzed using a Becton Dickenson FACScan. Apoptotic events were considered to be annexin V^+ and $PI^{-/+}$. The apoptosis-inducing activity of purified anti-HLA-DR antibodies was similarly determined using KG-1 or THP-1 cells.

c. Flow cytometry for cell line and blood cell reactivities

To determine blood cell reactivities of K8-355, peripheral blood from normal donors was stained with FITC-conjugated K8-355, L227, or L243 for 15 minutes on ice. Red blood cells were removed by the addition of FACS lysing solution (Becton Dickinson catalog # 349202) for 10 minutes at room temperature. After washing, cell surface bound antibody was detected using a Becton Dickenson FACScan.

To determine if K8-355 is a pan-anti-MHC class II antibody, CESS (myelomonocytic leukemia, American Type Culture Collection (ATCC)), Daudi (Burkitts lymphoma, ATCC), KG-1 (acute myelocytic leukemia, ATCC), Raji (Burkitt's lymphoma, ATCC), Ramos (Burkitt's lymphoma, ATCC), RL (non-Hodgkin's lymphoma, ATCC), and THP-1 (acute myelocytic leukemia, ATCC) were analyzed by flow cytometry. In addition, binding of K8-355 was assessed on 9 individual normal donors as described above.

e. Antibody variable region sequences determination

Total RNA was extracted from approximately 10^7 hybridoma cells using TRIzol reagent (Life Technologies, Gaithersburg, MD) and poly(A)$^+$ RNA was isolated with the PolyATract mRNA Isolation System (Promega, Madison, WI) according to the suppliers' protocols. Double-stranded cDNA was synthesized using the SMART^{TM}RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) following the supplier's protocol. The variable region cDNAs for the light and heavy chains were amplified by polymerase chain reaction (PCR) using 3' primers that anneal respectively to the mouse kappa and gamma chain constant regions, and a 5' universal primer provided in the SMART^{TM}RACE cDNA Amplification Kit. The 5' universal primer for VL has the sequence:

5' GAT GGA TAC AGT TGG TGC AGC-3' , and that for VH has the sequence:

5'-GCC AGT GGA TAG ACA GAT GG-3'.

For VL PCR, the 3' primer has the sequence:

5'-TATAGAGCTCAAGCTTGGATGGTGGGAAGATGGATACAGTTGGTGC-3'

with residues 17- 46 hybridizing to the mouse Ck region. For VH PCR, the 3' primers have the degenerate sequences:

5'-TATAGAGCTCAAGCTTCCAGTGGATAGAC(ACT)GATGGGG(GC)TGT(CT) GTTTTGGC-3'

with residues 17 - 50 hybridizing to mouse gamma chain CH1. The VL and VH cDNAs were subcloned into pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA) for sequence determination. DNA sequencing was carried out by PCR cycle sequencing reactions with fluorescent dideoxy chain terminators (Applied Biosystems, Foster City, CA) according

to the manufacturer's instructions. The sequencing reactions were analyzed on a Model 377 DNA Sequencer (Applied Biosystems).

f. Affinity Purification of Antigen

Cells (5 X 10^8) were lysed in 1% vol/vol NP-40, 0.5% wt/vol deoxycholate, 20 mM Tris HCl, pH 8.2, 150 mM NaCl, 1 mM EDTA, 25 μg/ml aprotinin, 25 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride for 1 hour on ice with frequent mixing. Lysate was centrifuged for 20 minutes at 300 x *g* to remove nuclei and debris. Antigens were purified by standard hybridoma affinity chromatography techniques as described in Hill et al, *J. Immunol.* 152, 2890-2898 (1994).

g. N-terminal Sequencing

Proteins to be sequenced were separated by SDS-PAGE on a 4-20% gradient gel and then electrotransferred onto a PVDF membrane. The membrane was stained for 2 minutes using colloidal Coomassie and then destained in water. The resulting bands were excised and subjected to N-terminal Edman sequencing as described by Miller, *Methods: A Companion to Methods in Enzymology* 6, 315 (1994).

h. Antigen Grouping

Cells (2 X 10^7) were cell surface iodinated as described (Landolfi and Cook, *Mol. Immunol.* 23, 297-309 (1986)). Alternately, cells were cell surface biotinylated with EZ-Link Sulfo-NHS-LC-Biotin per manufacturers protocol (Pierce cat # 21335). Cells were then lysed in 1% NP-40, 0.5% deoxycholate, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF for 1 hour on ice. Cell lysate was centrifuged at 14,000 x *g* for 5 minutes to remove nuclei and debris. Cell lysate was pre-cleared with rotation by incubation with Gamma Bind Plus Sepharose beads for 2 hours at 4 °C. The beads were spun down and the cell lysate was then aliquoted into Eppendorf tubes containing Gamma Bind Plus Sepharose beads that had been pre-incubated with hybridoma supernatant. The tubes were rotated overnight at 4 °C. After extensive washing, bound antigen was eluted from the beads by boiling in the presence of 5% wt/vol SDS, 125 mM Tris-HCl, pH 6.8, and 4% vol/vol β-mercaptoethanol, and 50% vol/vol glycerol. Proteins were then subjected to SDS-PAGE. After electrophoresis, the gel was fixed for 30 minutes with 60% H_2O/30% methanol/10% acetic acid. The gel was then washed for 30 minutes with water then dried down. The dried gel was put on film (Kodak® Biomax MS® film with appropriate Biomax MS® screen) overnight. For cell surface biotinylated samples, after electrophoresis proteins were electrotransferred onto a PVDF membrane. The membrane

was blocked for 1.5 hours at room temperature in Superblock (Pierce cat #37515). The membrane was then incubated with HRP-conjugated avidin in PBS with 1% BSA and 0.5% Tween-20 for 1 hour at room temperature. After extensive washing, the membrane was developed using enhanced chemiluminescence followed by fluorography.

RESULTS AND DISCUSSIONS

One BALB/c mouse was immunized with a lipid raft preparation from the acute myelogenous leukemia cell line KG-1. After two boosts, lymphocytes were isolated from the mouse lymph nodes and fused with myeloma NS0 cells to generate hybridomas according to the methods described in Example 3. A total of about 1142 hybridomas were generated and supernatant from each hybridoma was screened by flow cytometry for binding to KG-1. About 392 hybridomas tested positive. As the normal counter part of acute myelogenous leukemic cells are difficult to purify to screen for differentially expressed antigens, we opted to identify those monoclonal antibodies that have a more restricted expression in blood cells and have anti-cancer activity against KG-1. The 392 KG-1-positive hybridomas were screened against a human T cell line Jurkat by flow cytometry to eliminate those that were reactive to T cells. Antibodies from one hundred and four hybridomas that were Jurkat-negative were identified and their supernatants were tested for KG-1 cell death-inducing activity. Antibodies from 36 selected hybridomas that had apoptosis-inducing activity in an overnight assay were retested in various assays for further characterization. These additional assays included one for the antibody's apoptosis-inducing activity in another AML cell line (THP-1) and several for the antibody's binding activity to T cells, red blood cells, platelets, granulocytes, stem cells (CD34+), lymphocytes and monocytes by flow cytometry. A flow chart of how these 36 anti-KG-1 hybridomas were selected is shown in Figure 17. The reactivity profiles of these 36 anti-KG-1 hybridomas were summarized in Table 4.

Antigen grouping by immunoprecipitation as demonstrated in Example 3 was used to reduce the number of tumor-associated antigens that need to be characterized. The molecular weights of 24 antigens were determined (Figure 18) and summarized in Table 4. Fifteen antibodies immunoprecipitated an antigen consisting of two chains of 32 kD and 28 kD. The molecular weights of these two chains resemble those of major histocompatibility complex class II antigen HLA-DR, a predominant antigen in blood cells. We also know by previous experience that some anti-HLA-DR antibodies have potent apoptosis-inducing activity against lymphoma and leukemic cells (Kostelny et al.

Int. J. Cancer 93, 556-565 (2001)). Using antibody from K8-355 as a representative of this group, we made an affinity column to isolate the antigen from KG-1 cells and confirmed its identity to be HLA-DR by MALDI-TOF peptide profiling.

Data indicate that the antibody K8-355 (murine IgG1/kappa) has a pan-HLA-DR reactivity; it binds to all cells that express HLA-DR in spite of the polymorphic nature of the molecules. The variable regions of the antibody K8-355 have also been determined (Figures 19 and 20). In addition to having apoptosis-inducing activity against AML cells such as KG-1 and THP-1, K8-355 also has apoptosis-inducing against the B cell lines Raji and Daudi (Figure 21). These data indicate that pan-HLA-DR antibodies may have clinical application against leukemia and lymphoma.

Example 5

This example describes determining the identity of lipid raft tumor-associated antigens.

Monoclonal antibody that binds to each tumor-associated antigen is to be purified from hybridoma spent medium by protein-G affinity chromatography. The purified monoclonal antibody is then covalently linked to CNBr-activated Sepharose resin to generate an affinity column for a particular antigen. LNCaP or KG-1 cell lysate is to be prepared as in the immunoprecipitation experiment and passed onto the affinity column. Antigen retained in affinity column is eluted and subjected to protein sequence determination by the Edman degradation method. The determined N-terminal sequence is used to search for gene product identity against the Human Genome data bank. Alternatively, the eluted antigen can be subjected to MALDI-TOF peptide-mass profiling and the derived fingerprints be used to search for protein identity against the Human Genome data bank.

Example 6

This example describes inhibition of tumor adhesion and spreading by cell adhesion and migration assay

Antibody inhibition of adhesion and spreading is evaluated. Tissue culture 12-well plates were coated 2 hours at room temperature with components of the extracellular matrix, i.e. vitronectin (VN), fibronectin (FN), collagen type I, type III and type IV, laminin (LA), or hyaluronic acid (HA) in Hanks buffered salt solution (HBSS). Plates are blocked for 2 hours with 1% BSA in PBS. Cells are plated in HBSS with 1 mM $CaCl_2$

and 1 mM $MgCl_2$ in the presence or absence of antibody. Cells are allowed to spread for 30 minutes to 2 hours at 37 °C prior to photography.

Inhibition of cancer cell migratory activity of anti-tumor agents is evaluated in a matrigel assay. Membranes with a pore size of 8 μm were coated with 50 μl matrigel. The membranes were inserted into 24 well plates that contain medium without supplements. Cancer cells are resuspended in medium with 10% FCS in the presence or absence of antibodies and then seeded on the matrigel coated transwell plates. Plates are incubated for 48 hours at 37 °C. Thereafter, cells at the bottom of the chamber are counted using an inverted microscope.

Example 7

This example describes using xenograft models to test the efficacy of the anti-tumor agents.

For solid tumor models, such as LNCaP xenograft, six to ten week old male nude NCR nu/nu mice are inoculated subcutaneously in the mid-scapular region with 5 X 10^6 androgen-dependent LNCaP cells. Cells that are injected are reconstituted with basement membrane in the form of Matrigel as described (Sato et al *Cancer Res.* 5, 1584-1589 (1997)). To maintain serum testosterone levels, male mice are implanted with 12.5 mg sustained release testosterone pellets subcutaneously prior to receiving the tumor cell inoculation. Antibodies against ATP synthase, or specific for LNCaP lipid raft antigens are given intraperitoneally on day 2 and 4. Tumors are measured every three to four days with vernier calipers. Tumor volumes are calculated by the formula $\pi/6$ X (larger diameter) X (smaller diameter)^2. For the androgen-independent prostate cancer xenograft studies, DU 145 or PC-3 cells are used. For the hematological tumor model, such as KG-1 xenograft, a similar approach may also be tried (Dao, et al., Curr. Opin. Mol. Ther. 1=553-7, 1999)

Example 8

This example describes selection of anti-lipid raft antibodies for cancer therapy based on their antigen expression profiles and anti-cancer activities *in vitro*.

For solid tumors, monoclonal antibodies against the identified antigens are used to stain by immunohistochemistry normal or neoplastic human tissues to establish the expression profiles of the tumor associated antigens. Valuable tumor-associated tumor antigens should have low or no expression in normal tissues and high expression in cancer cells. To be a good targets for antibody therapy, tumor associated antigens should

be differentially expressed in substantial percentage (20% and above) of certain cancer type. Valuable antibodies against these antigens may have anti-cancer activities *in vitro*. These activities include inhibition of cell proliferation, induction of apoptosis and inhibition of cell migration.

For hematological malignancies, monoclonal antibodies against the identified antigens are used to stain by flow cytometry patient's leukemic cell as well as normal human blood and bone marrow cells. Antigens that are expressed in hematopoietic stem cells (within the CD34-positive population), T cells, platelets, or granulocytes should be excluded because triggering or killing of these cells by antibodies will cause severe toxicity in humans. Antigens of interest may be expressed in B cells, macrophages or monocytes but not in other normal tissues. The ideal tumor-associated antigens are the ones that can be triggered to induce cell death in leukemic cells.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention.

All publications, patents, patent applications, and web sites are herein incorporated by reference in their entirety to the same extent as if each individual patent, patent application, or web site was specifically and individually indicated to be incorporated by reference in its entirety.

Claims.

1. A method for identifying a tumor target comprising examining a lipid raft, wherein said lipid raft is derived from a tumor cell or a normal cell.

2. The method according to Claim1, wherein said examining comprises:

   a. isolating lipid rafts from a tumor cell and a normal cells;

   b. comparing the lipid raft protein expressions of said tumor cell and said normal cell;

   c. isolating a protein that is differentially expressed in said tumor cell.

3. The method according to Claim 2, further comprising:

   d. identifying partial or full amino acid sequence of said molecule, or partial or full nucleic acid sequence encoding said isolated protein.

4. The method of identifying an anti-tumor agent comprising selecting an inhibitor of said molecule according to Claim 2.

5. A method for identifying anti-tumor targets comprising:

   a. isolating lipid rafts;

   b. separating the lipid rafts by mean of electrophoresis, so that individual protein bands are separated from each other;

   c. comparing the protein expressions of said lipid rafts from cancer cells and from normal cells.

   d. isolating a protein band that is differentially expressed in cancer cells; and

   e. identifying partial or full amino acid sequence of the protein, or partial or full nucleic acid sequence encoding the protein.

6. The method according to Claim 3, wherein said tumor target is a prostate tumor target, wherein said tumor cell is a prostate tumor cell and said normal cell is a normal prostate cell.

7. A method of identifying anti-prostate tumor agents comprising selecting an inhibitor of said prostate tumor target according to Claim 6.

8. A method for generating an antibody against a tumor target associated with a type of tumor cells, comprising:

a. isolating lipid rafts from said type of tumor cells; and

b. immunizing an animal host by said isolated lipid rafts.

9. The method according to Claim 8 further comprising:

c. producing hybridomas from the immunized animal host, wherein said hybridomas produce monoclonal antibodies;

d. selecting said monoclonal antibodies; and

e. purifying said selected monoclonal antibodies.

10. A method according to Claim 9, wherein said selecting comprises selecting monoclonal antibodies that bind to said type of tumor cells but not to normal cells.

11. A method according to Claim 10, wherein said selecting further comprises selecting monoclonal antibodies that induces apoptosis of said type of tumor cells.

12. A method according to Claim 9, where said selecting comprising selecting monoclonal antibodies that inhibit cell proliferation of said type of tumor cell.

13. A method according to Claim 10, wherein said type of tumor cells are prostate tumor cells, said normal cells are non-cancerous prostate cells.

14. A method according to Claim 11, wherein said type of tumor cells are leukemia cells, said normal cells are T cells.

15. A method of identifying a tumor target comprising identifying an antigen that binds to the selected antibodies according to Claim 9, wherein said identifying comprises identifying a partial or full amino acid or nucleic acid of said antigen.

16. A hybridoma produced by the method according to Claim 13.

17. A hybridoma produced by the method according to Claim 14.

18. An antibody generated by the hybridoma according to Claim 16.

19. An antibody generated by the hybridoma according to Claim 17.

20. An antigen that binds to the antibody according to Claim 18 or 19.

21. An isolated lipid raft derived from a prostate tumor cell, wherein said isolated lipid raft comprises a polypeptide that is differentially expressed in a prostate tumor cell.

22. The isolated lipid raft according to Claim 21, wherein said isolated lipid raft is clustered with other lipid rafts derived from said prostate tumor cell.

23. The isolated lipid raft according to Claim 21, wherein said isolated lipid raft is a detergent resistant membrane (DRM).

24. The isolated lipid raft according to Claim 21, wherein said polypeptide is selected from the group consisting of PMSA, CD10, Trop-1, ATP synthase, NCAM2, and CD222.

25. A monoclonal antibody that binds to the isolated lipid raft according to Claim 21.

26. The monoclonal antibody according to Claim 25, wherein said monoclonal antibody binds to or neutralizes PMSA.

27. The monoclonal antibody according to Claim 25, wherein said monoclonal antibody binds to or neutralizes CD10.

28. The monoclonal antibody according to Claim 27, wherein said monoclonal antibody specifically binds to prostate cancer cells but not binds to other types of cancer cells.

29. The monoclonal antibody according to Claim 25, wherein said monoclonal antibody binds to or neutralizes Trop-1.

30. The monoclonal antibody according to Claim 29, wherein said monoclonal antibody reduces the colony formation of prostate cancer cells by more than 60%.

31. The monoclonal antibody according to Claim 25, wherein said monoclonal antibody binds to or neutralizes ATP synthase.

32. The monoclonal antibody according to Claim 25, wherein said monoclonal antibody binds to or neutralizes NCAM2.

33. The monoclonal antibody according to Claim 25, wherein said monoclonal antibody binds to or neutralizes CD222.

34. An isolated lipid raft derived from a leukemia cell, wherein said isolated lipid raft comprising a polypeptide that is differentially expressed in said leukemia cell compared to a normal T cell.

35. The isolated lipid raft according to Claim 34, wherein said leukemia cell is a KG-1 cell.

36. A monoclonal antibody that binds to the isolated lipid raft and the polypeptide according to Claim 35.

37. The antibody according to Claim 36 wherein said antibody induces apoptosis of said leukemia cell.

38. The isolated lipid raft according to Claim 35, wherein said polypeptide is HLA-DR antigen.

39. The antibody according to Claim 38, wherein said antibody induces apoptosis of said tumor cells by more than 80%.

40. A method of treating prostate cancer comprising administering into a subject in need of such a treating a pharmaceutically effective amount of the antibodies according to Claim 25, 26, 28, 30, 32, or 33.

41. A pharmaceutical composition comprising the pharmaceutical carrier and the antibodies according to Claim 25, 26, 28, 30, 32, or 33.

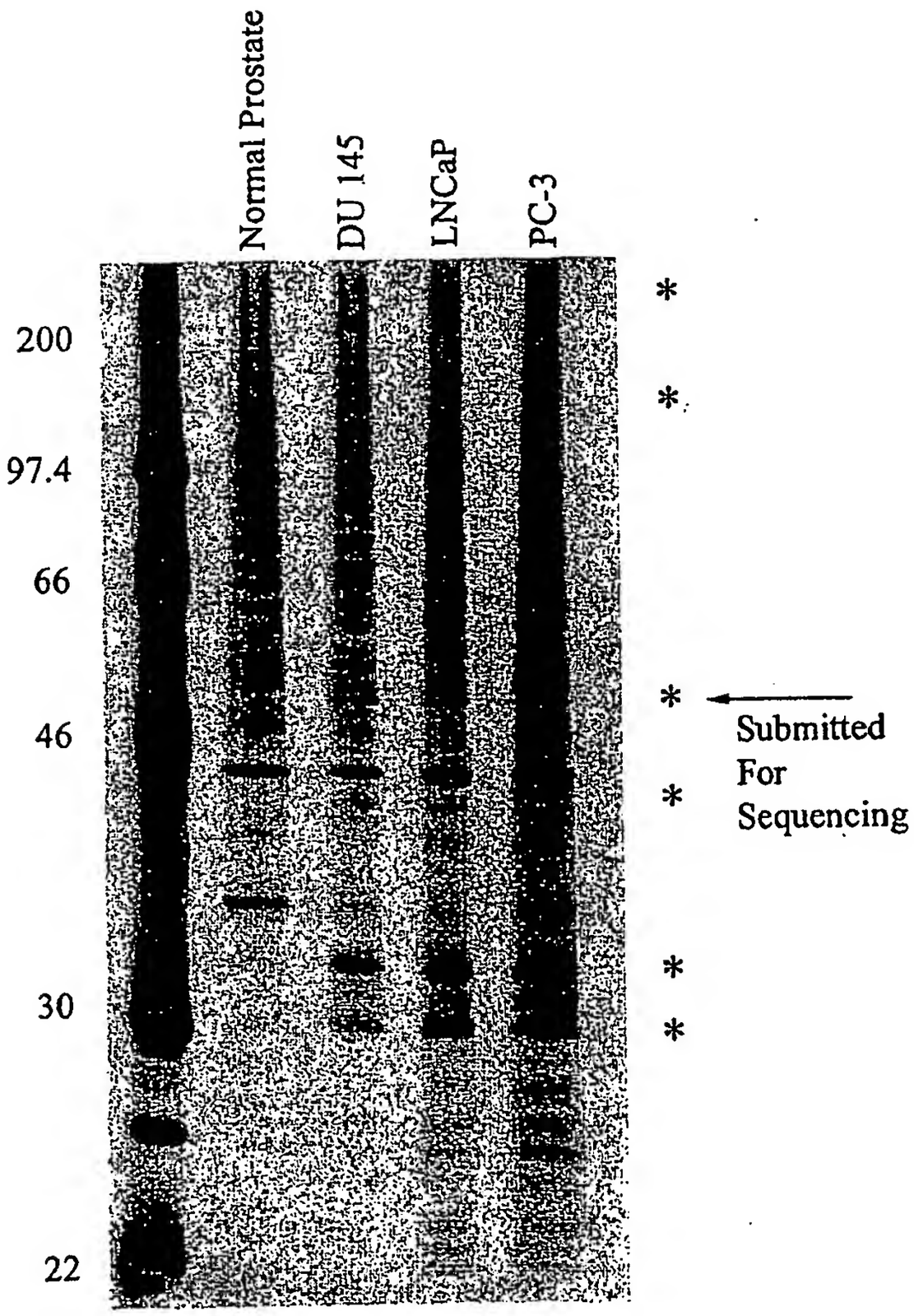

Prostate
Normal
DU 145
LNCaP
PC-3
200
97.4
66
46
30
22
Submitted
For
Sequencing

Figure 1

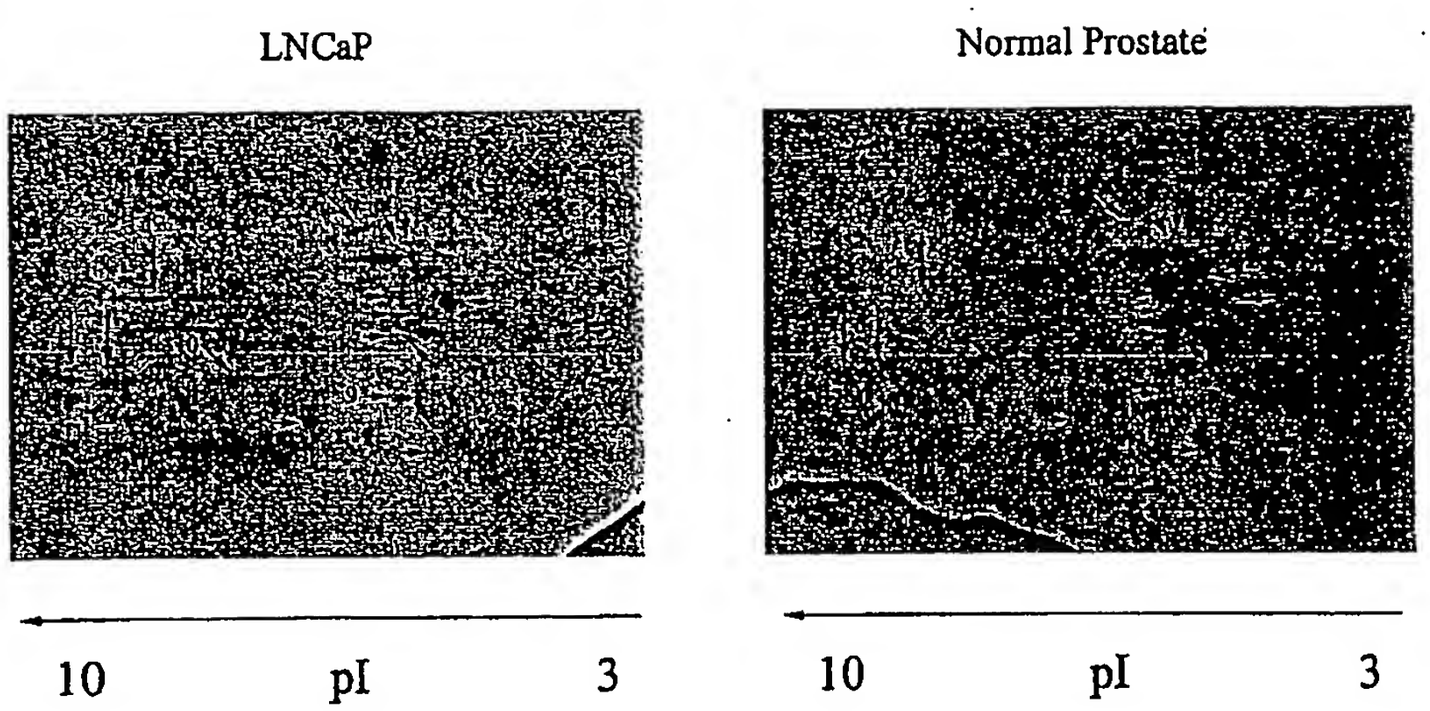


Figure 2

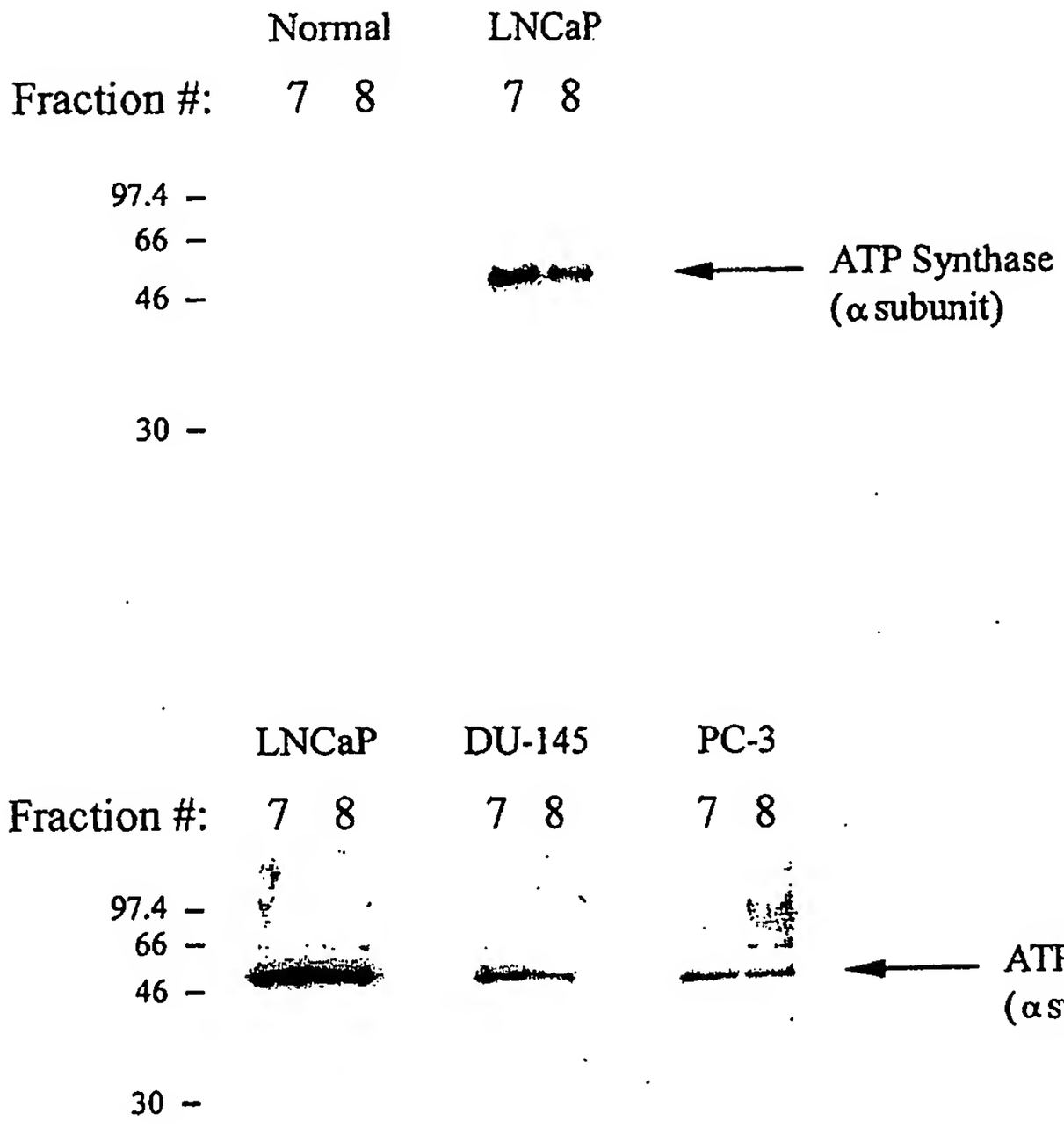

Normal
LNCaP
Fraction #:
7 8
7 8
97.4 –
66 –
46 –
30 –
ATP Synthase
(α subunit)
LNCaP
DU-145
PC-3
Fraction #:
7 8
7 8
7 8
97.4 –
66 –
46 –
30 –
ATP Synthase
(α subunit)

Figure 3

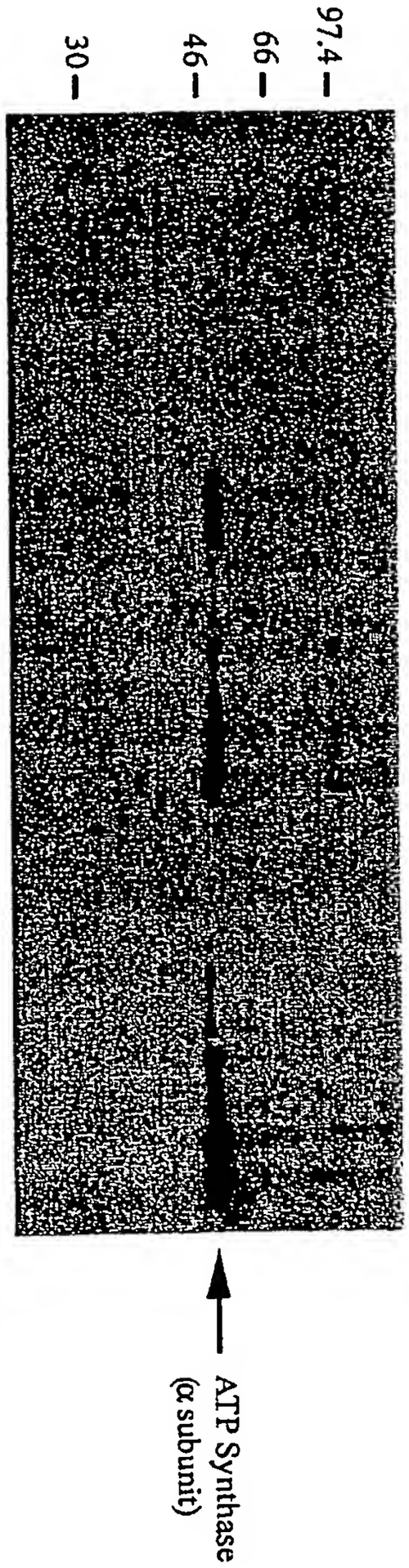

97.4
66
46
30
ATP Synthase
(α subunit)
BeWo (choriocarcinoma)
Colo205 (colorectal adenocarcinoma)
HT29 (colorectal adenocarcincoma)
JEG-3 (choriocarcinoma)
KG-1 (AML)
LS 180 (colorectal adenocarcinoma)
MCF7 (breast adenocarcinoma)
LNCaP (prostate carcinoma)
NCI-H292 (lung carcinoma)
PANC-1 (pancreas carcinoma)
RT4 (bladder carcinoma)
THP-1 (AML)
LNCaP (prostate carcinoma)

Figure 4

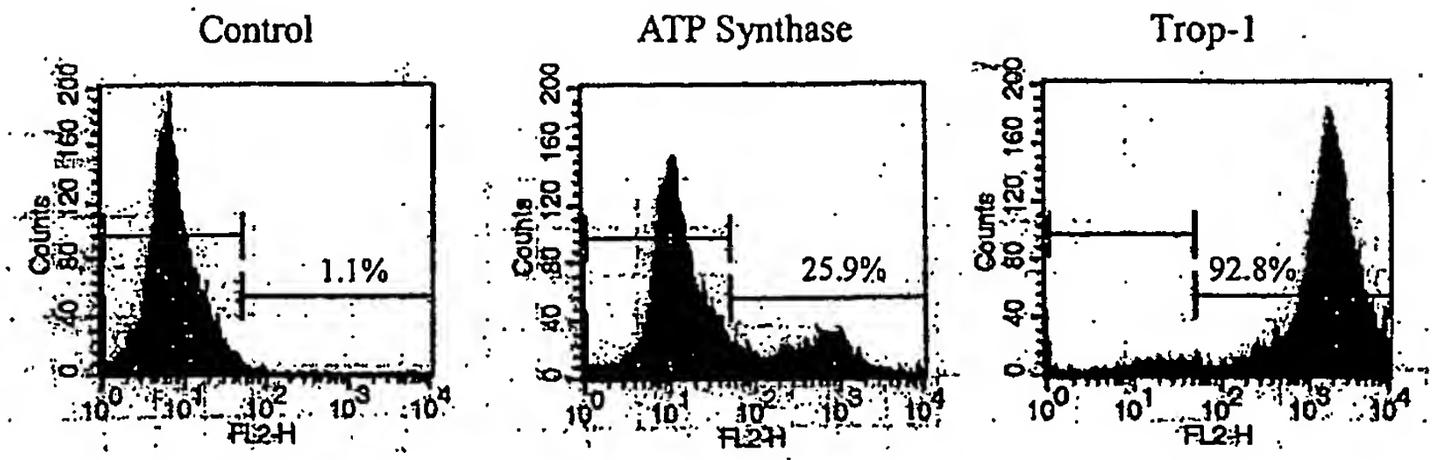


Figure 5

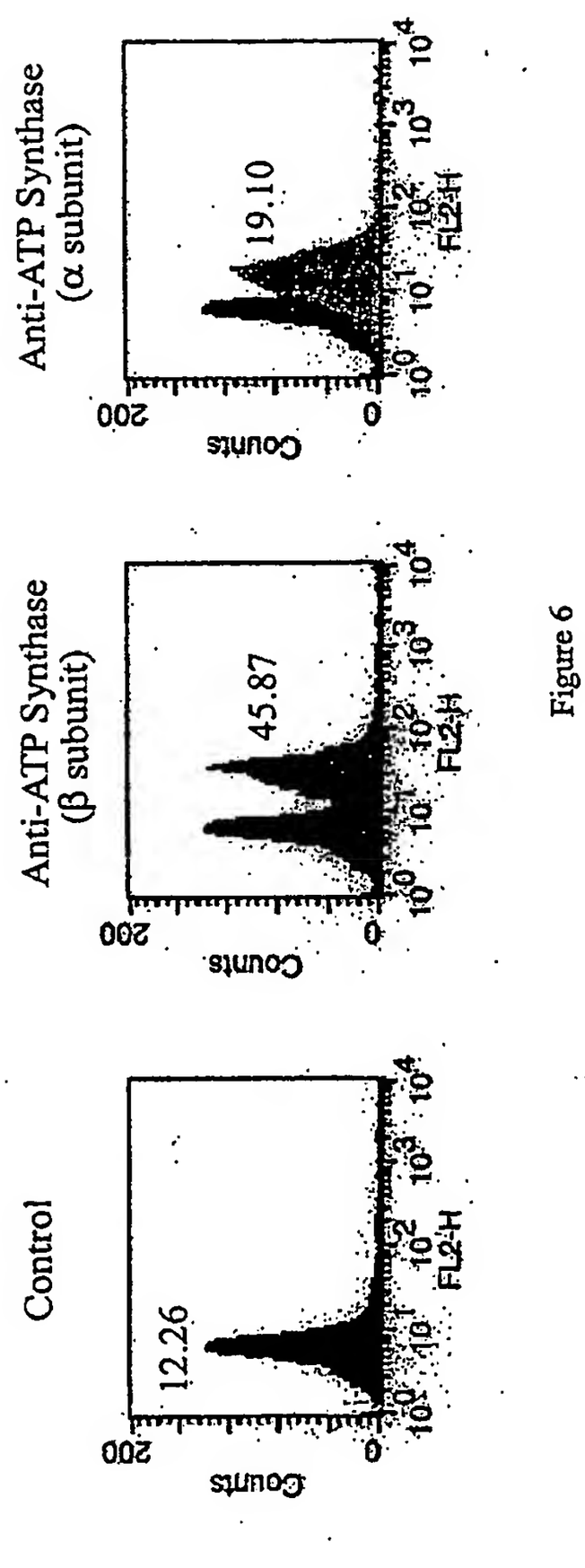
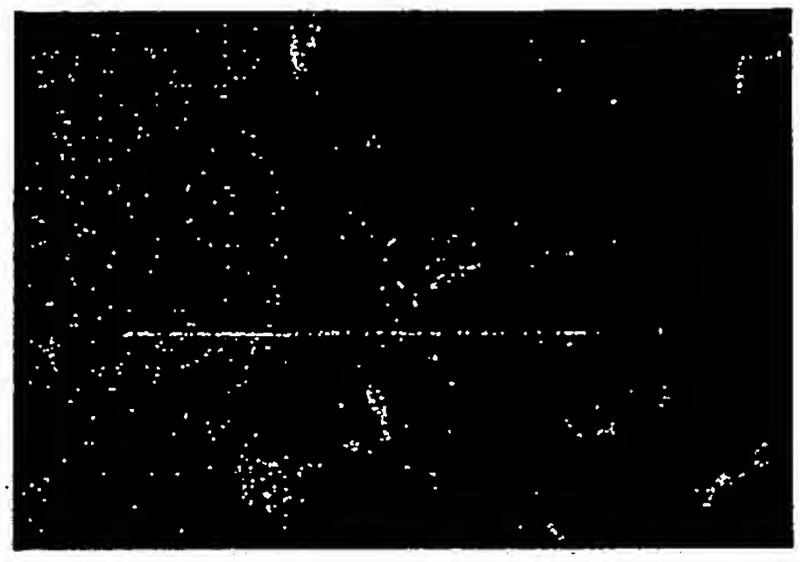

Anti-ATP Synthase
(α subunit)
19.10
Counts
200
0
FL2-H
Anti-ATP Synthase
(β subunit)
45.87
Counts
200
0
FL2-H
Control
12.26
Counts
200
0
FL2-H

Figure 6

anti-ATP synthase



anti-Trop 1

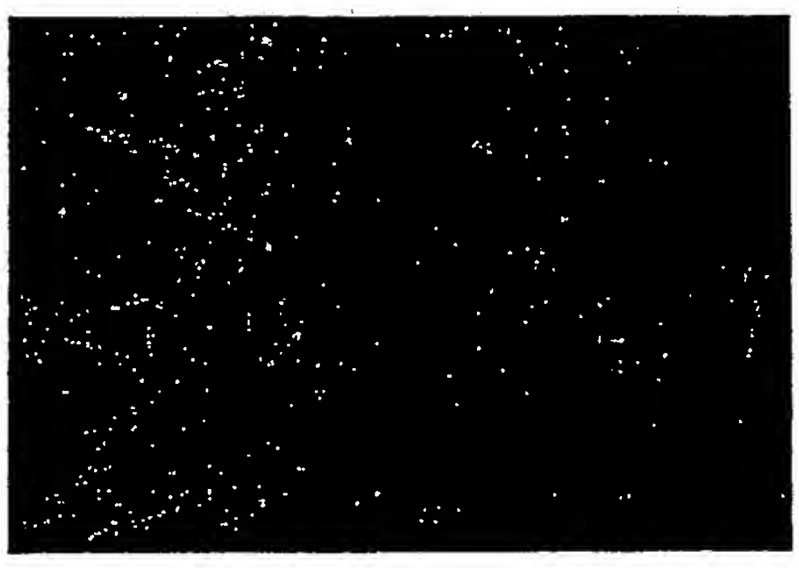


Figure 7

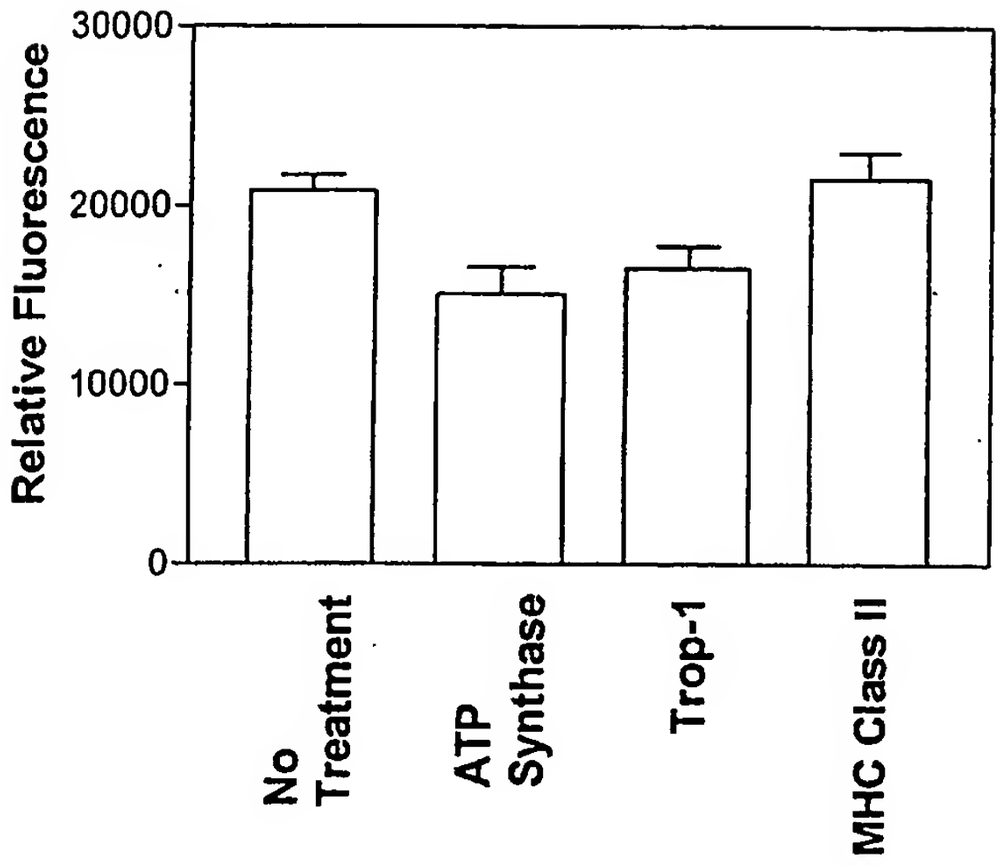

Relative Fluorescence
30000
20000
10000
0
No Treatment
ATP Synthase
Trop-1
MHC Class II

Figure 8

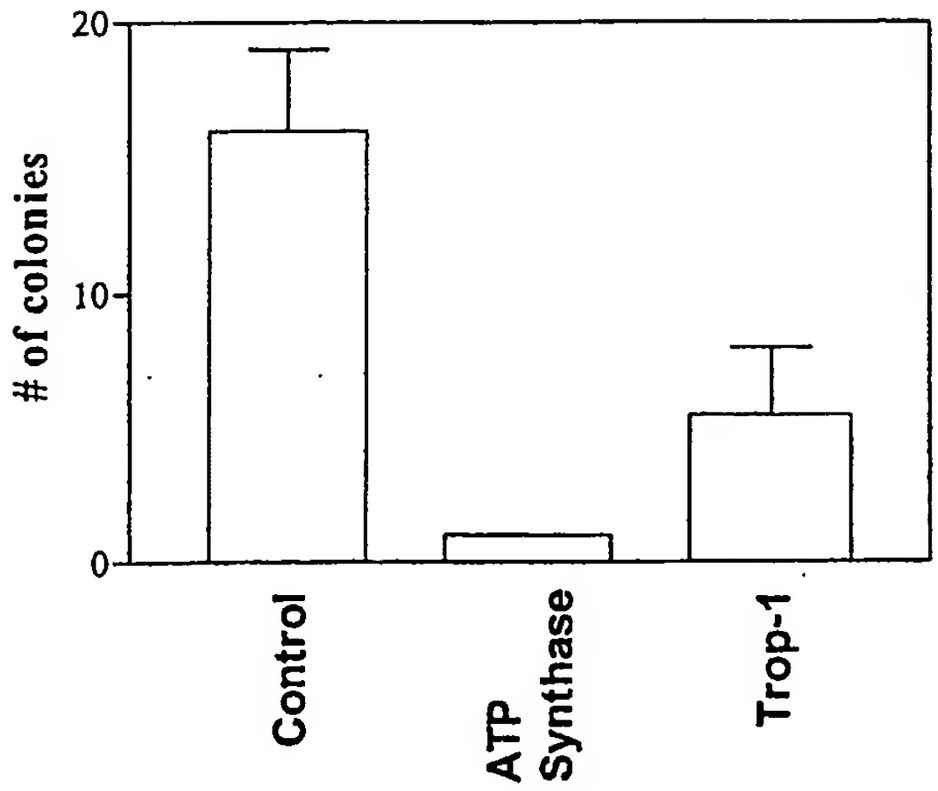


Figure 9

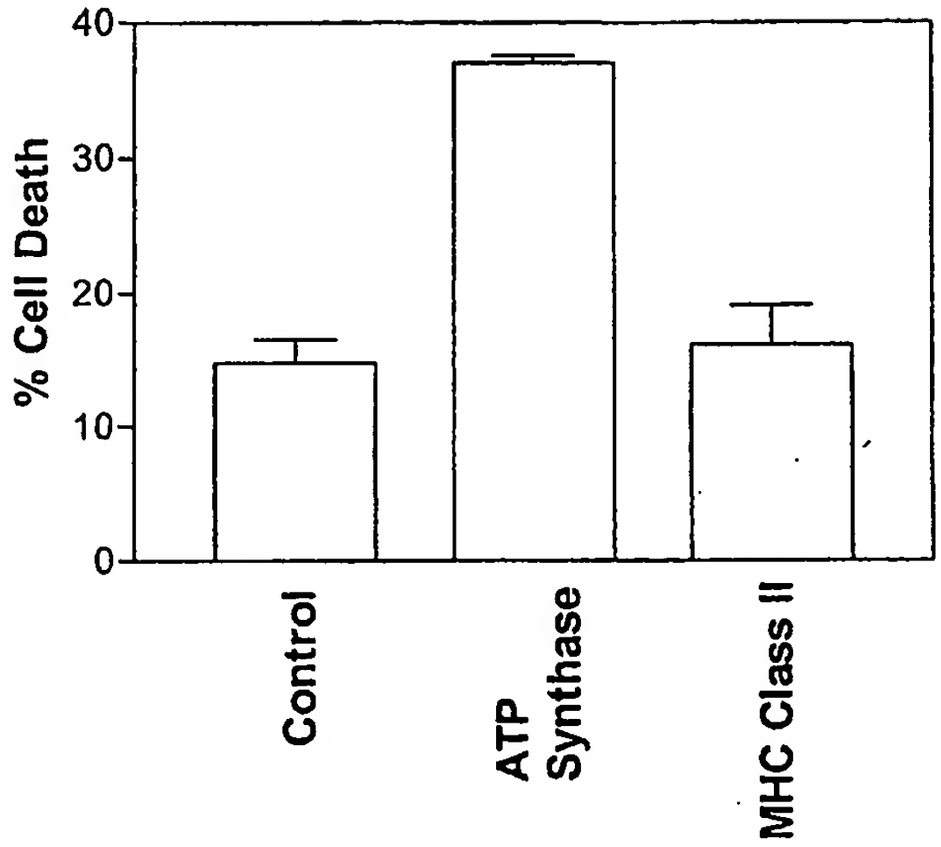


Figure 10

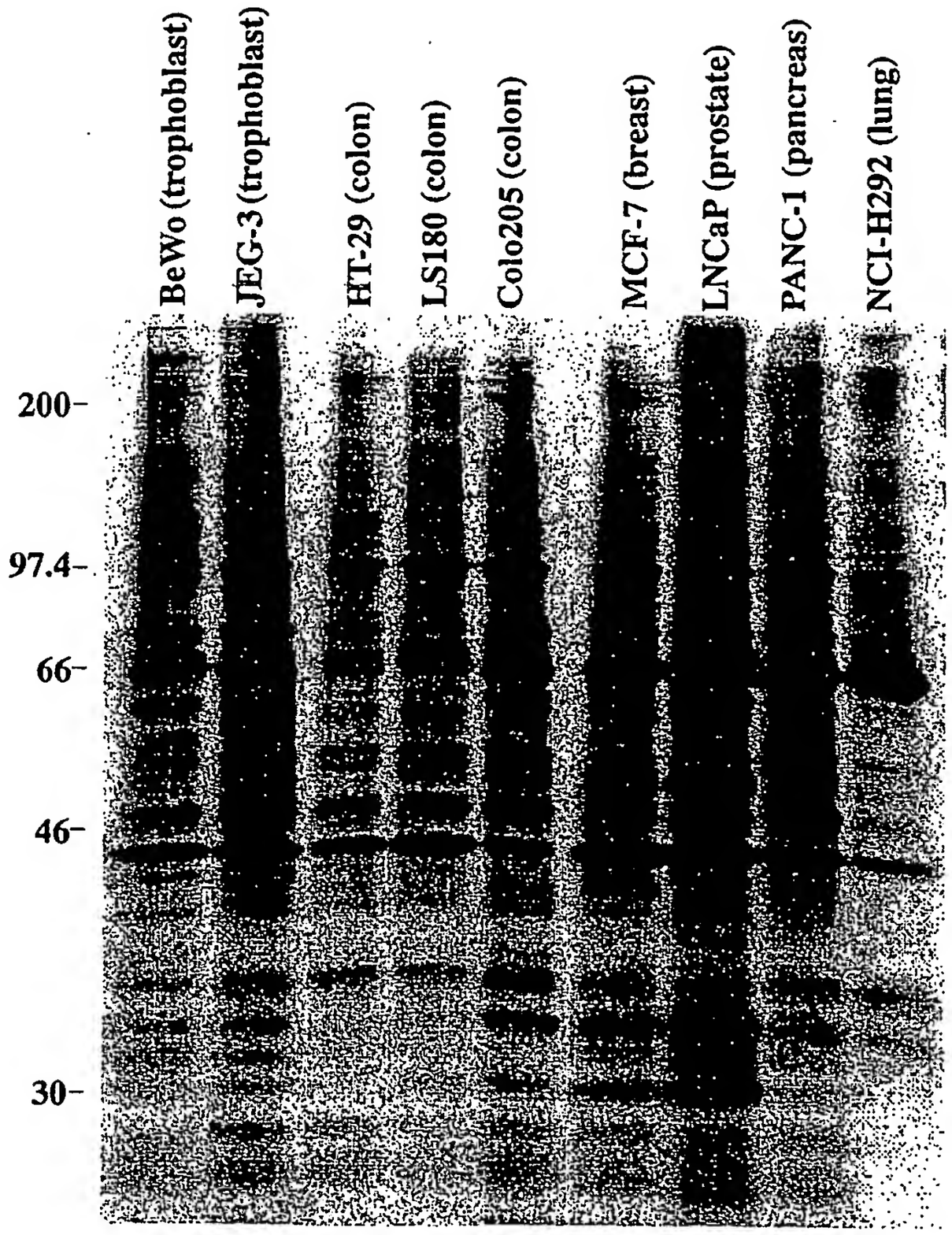

BeWo (trophoblast)
JEG-3 (trophoblast)
HT-29 (colon)
LS180 (colon)
Colo205 (colon)
MCF-7 (breast
LNCaP (prostate)
PANC-1 (pancreas)
NCI-H292 (lung)
200–
97.4–
66–
46–
30–

Figure 11

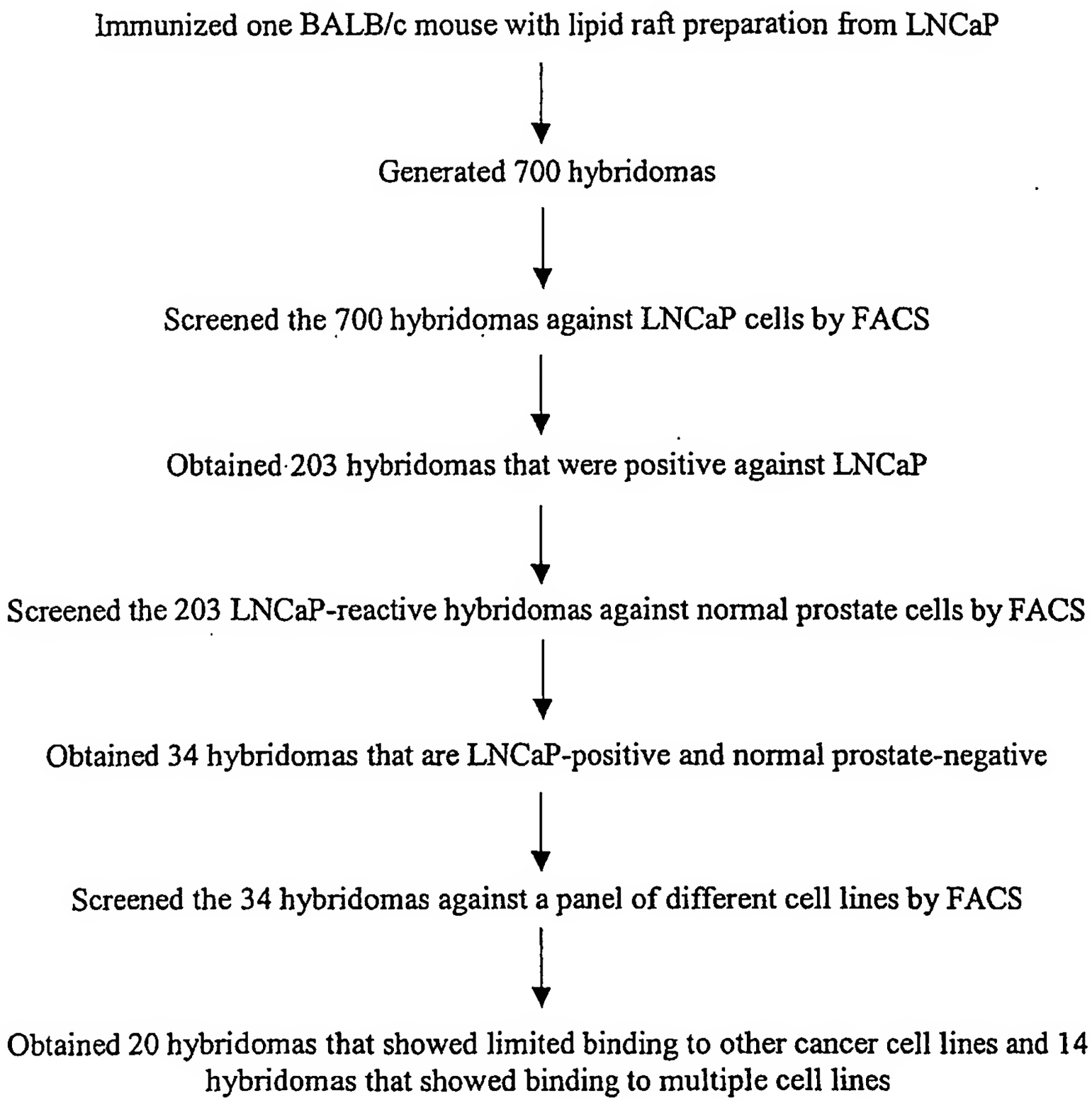

Immunized one BALB/c mouse with lipid raft preparation from LNCaP
Generated 700 hybridomas
Screened the 700 hybridomas against LNCaP cells by FACS
Obtained 203 hybridomas that were positive against LNCaP
Screened the 203 LNCaP-reactive hybridomas against normal prostate cells by FACS
Obtained 34 hybridomas that are LNCaP-positive and normal prostate-negative
Screened the 34 hybridomas against a panel of different cell lines by FACS
Obtained 20 hybridomas that showed limited binding to other cancer cell lines and 14 hybridomas that showed binding to multiple cell lines

Figure 12

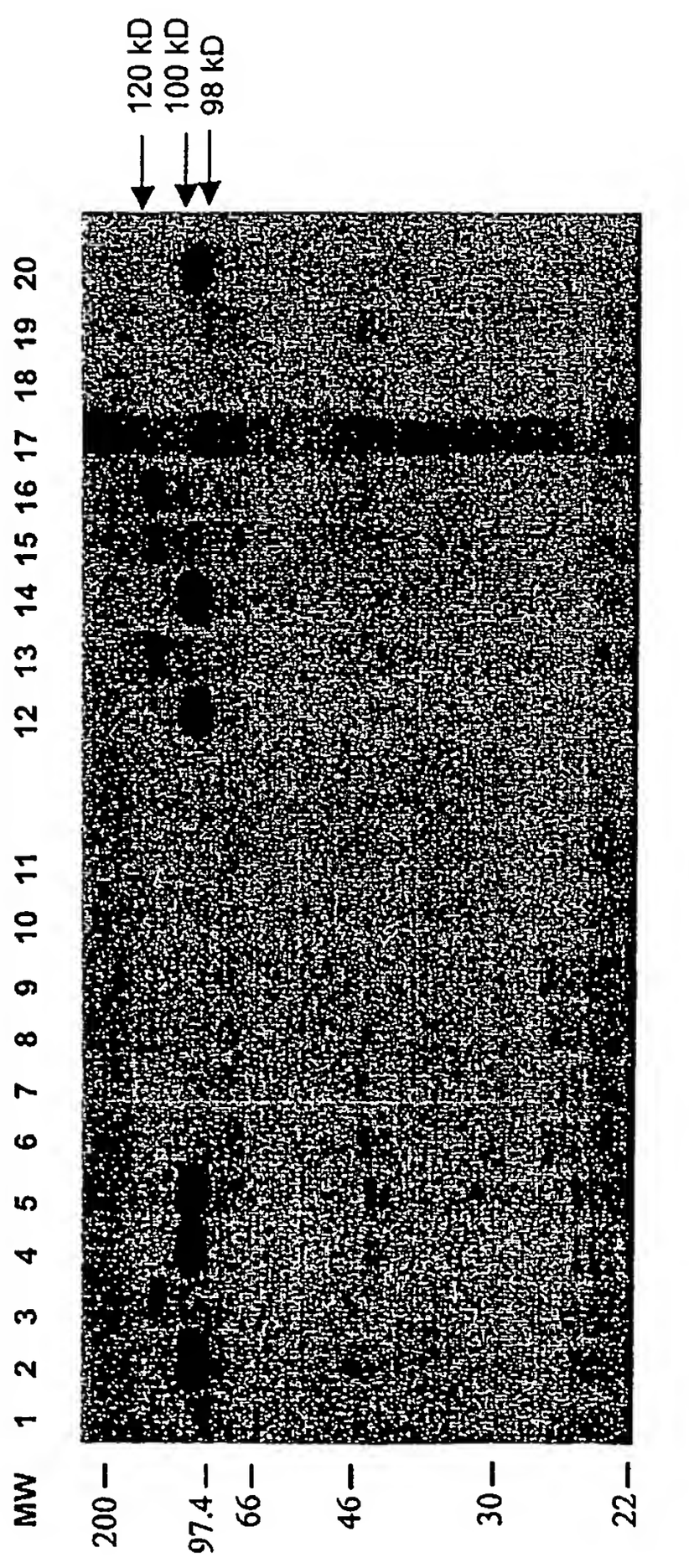

MW
200
97.4
66
46
30
22
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
120 kD
100 kD
98 kD

Figure 13

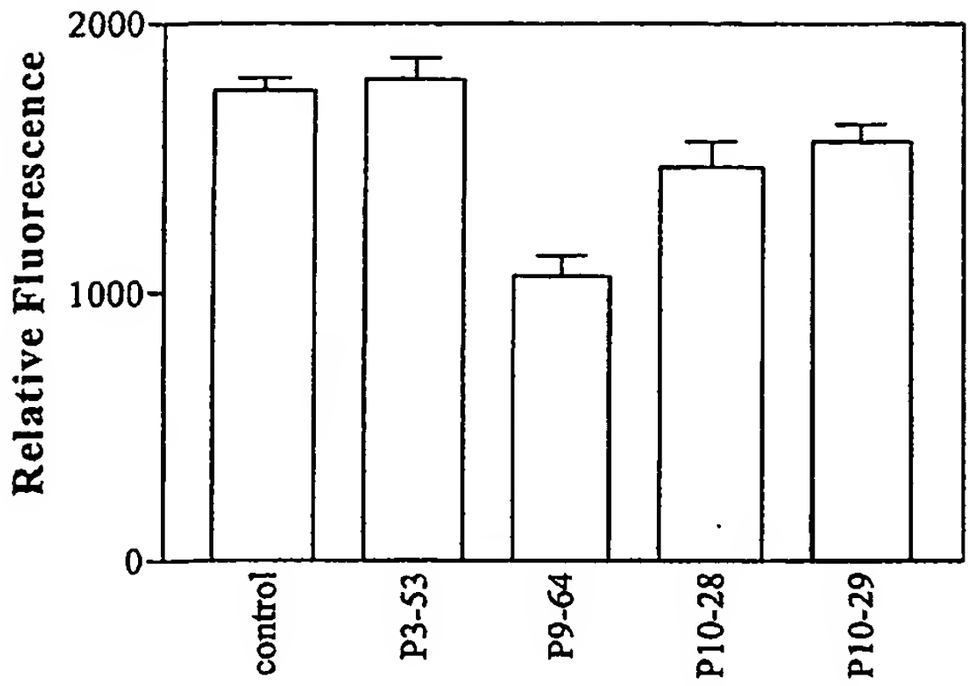

Relative Fluorescence
2000
1000
0
control
P3-53
P9-64
P10-28
P10-29

Figure 14

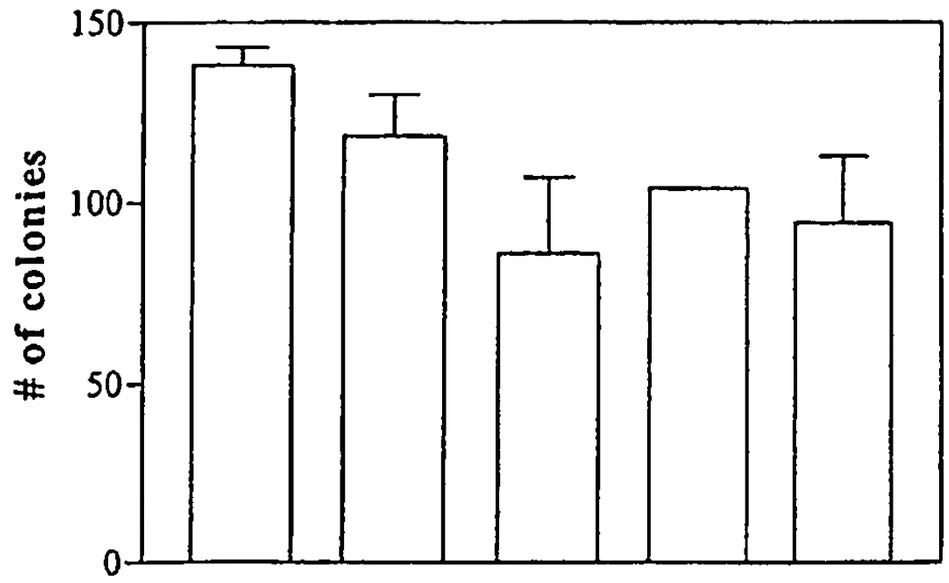


Figure 15

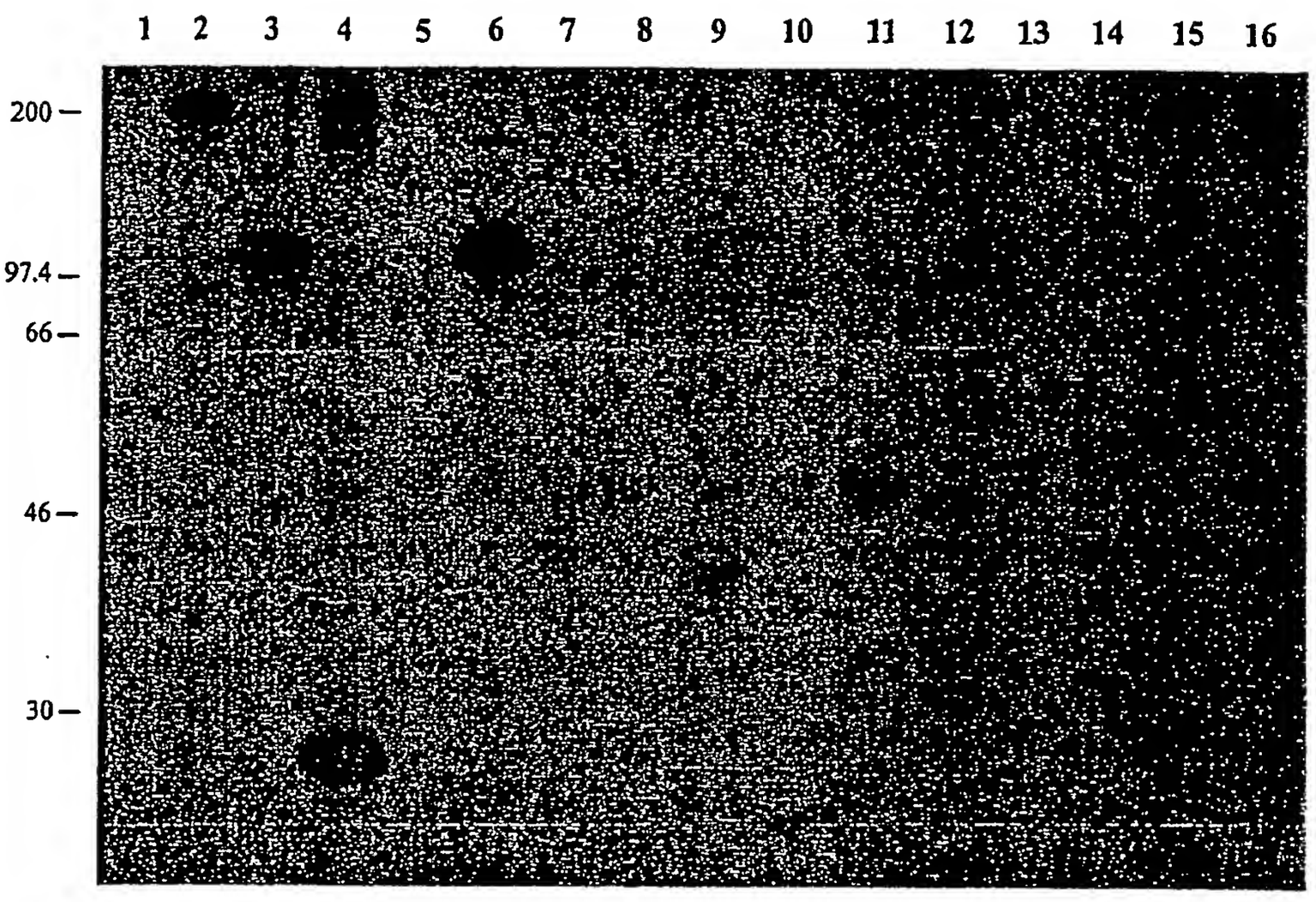


Figure 16

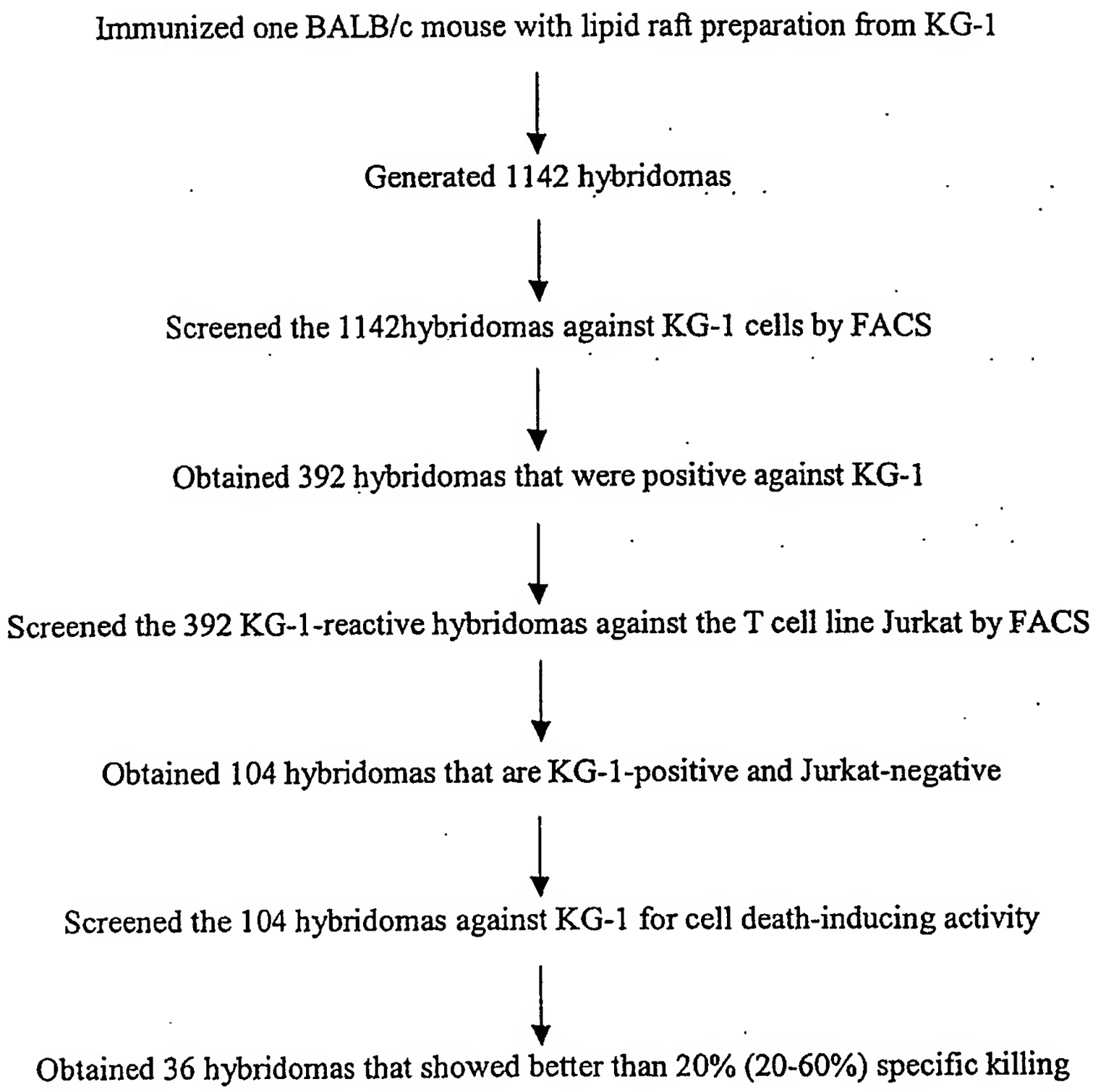

Immunized one BALB/c mouse with lipid raft preparation from KG-1
Generated 1142 hybridomas
Screened the 1142hybridomas against KG-1 cells by FACS
Obtained 392 hybridomas that were positive against KG-1
Screened the 392 KG-1-reactive hybridomas against the T cell line Jurkat by FACS
Obtained 104 hybridomas that are KG-1-positive and Jurkat-negative
Screened the 104 hybridomas against KG-1 for cell death-inducing activity
Obtained 36 hybridomas that showed better than 20% (20-60%) specific killing

Figure 17

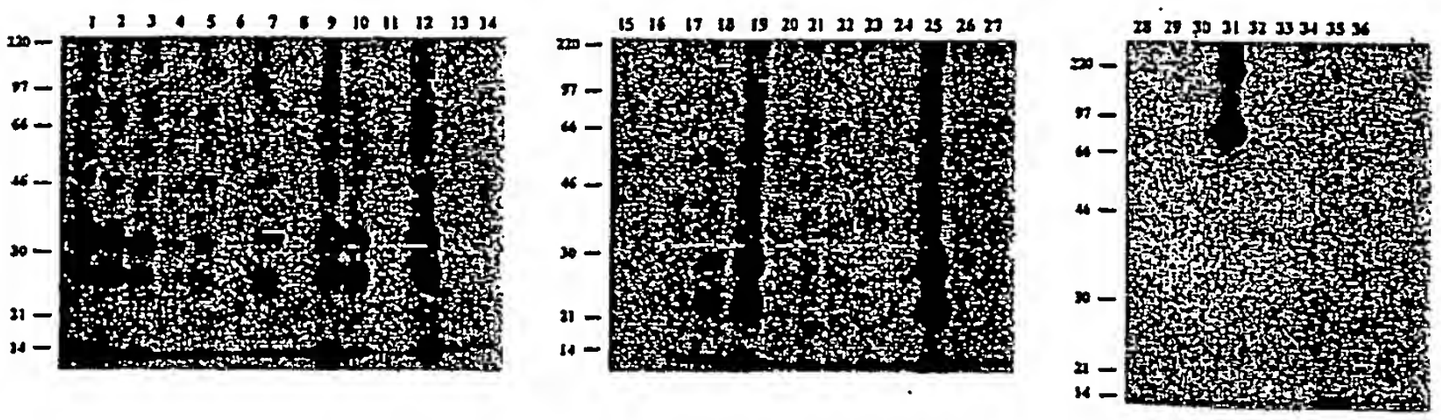


Figure 18 A

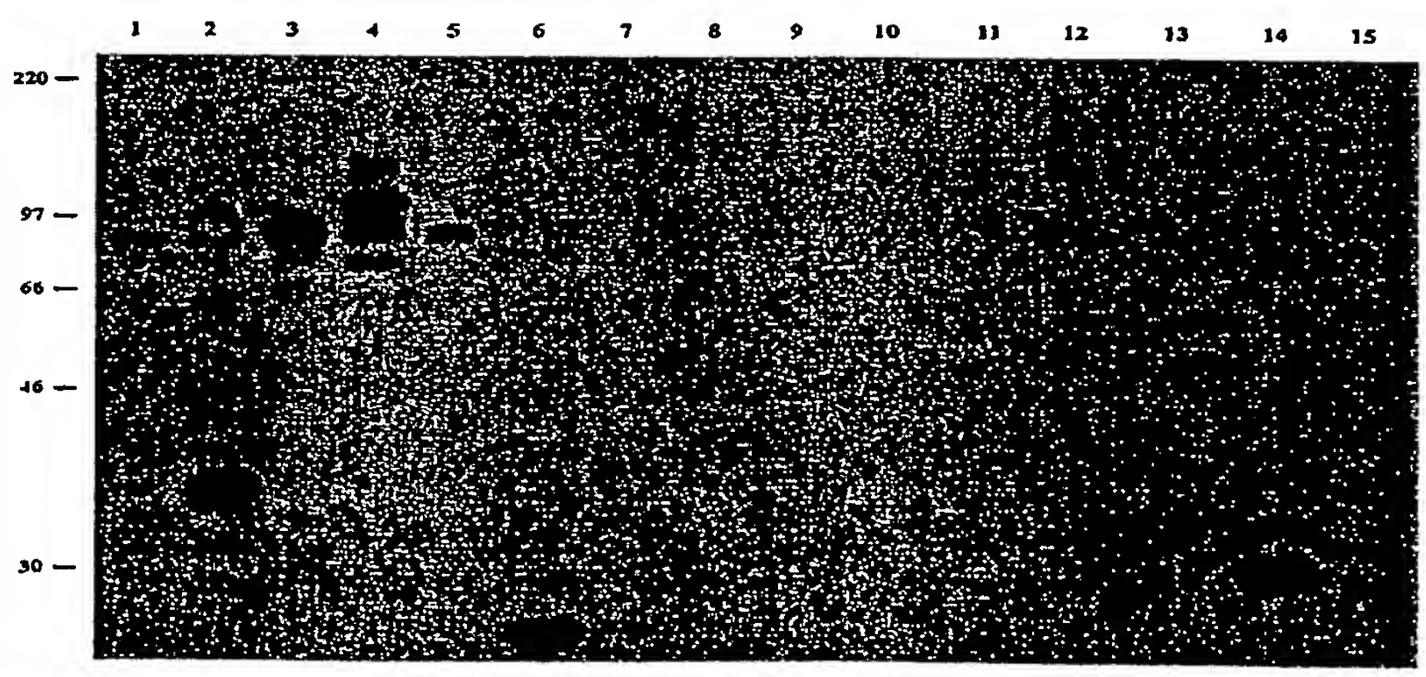


Figure 18 B

K8-355 Heavy chain variable region cDNA sequence

```
                                30                                  60
ATGGATTGGCTGTGGAACTTGCTATTCCTGATGGCAGCTGCCCAAAGTGCCCAAGCACAG
 M  D  W  L  W  N  L  L  F  L  M  A  A  A  Q  S  A  Q  A  Q

                                90                                 120
ATCCAGTTGGTGCAGTCTGGACCTGAGCTGAAGAAGCCTGGAGAGACAGTCAAGATCTCC
 I  Q  L  V  Q  S  G  P  E  L  K  K  P  G  E  T  V  K  I  S

                               150                                 180
TGCAAGGCTTCTAAATATACCTTCACAAACTATGGAATGAACTGGGTGAAGCAGGCTCCA
 C  K  A  S  K  Y  T  F  T  N  Y  G  M  N  W  V  K  Q  A  P

                               210                                 240
GGAAAGGTTTTAAGGTGGATGGGCTGGATAAACACCTACACTGGAGAGCCAACATATGCT
 G  K  V  L  R  W  M  G  W  I  N  T  Y  T  G  E  P  T  Y  A

                               270                                 300
GATGACTTCAAGGGACGATTTGCCTTCTCTTTGGAAACCTCTGCCAGCACTGCCTATTTG
 D  D  F  K  G  R  F  A  F  S  L  E  T  S  A  S  T  A  Y  L

                               330                                 360
CAGATCAACAACCTCAAAAATGAGGACATGGCTACATATTTCTGTGCAACGACTACTTTG
 Q  I  N  N  L  K  N  E  D  M  A  T  Y  F  C  A  T  T  T  L

                               390                                 414
ATTACTTACTACTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
 I  T  Y  Y  F  D  Y  W  G  Q  G  T  T  L  T  V  S  S
```

Figure 19

K8-355 Light chain variable region cDNA sequence

```
                                   30                                  60
ATGATGTCCTCTGCTCAGTTCCTTGGTCTCCTGTTGCTCTGTTTTCAAGGTACCAGATGT
 M  M  S  S  A  Q  F  L  G  L  L  L  L  C  F  Q  G  T  R  C

                                   90                                 120
GATATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTCTGGGAGACAGAGTCACC
 D  I  Q  M  T  Q  T  T  S  S  L  S  A  S  L  G  D  R  V  T

                                  150                                 180
ATCAGTTGCAGGTCAAGTCAGGACATTAGCAAATATTTAAACTGGTATCAGCAGAAACCA
 I  S  C  R  S  S  Q  D  I  S  K  Y  L  N  W  Y  Q  Q  K  P

                                  210                                 240
GATGGAACTGTTAAACTCCTGATCTACTACACATCAAGATTACACTCAGGAGTCCCATCA
 D  G  T  V  K  L  L  I  Y  Y  T  S  R  L  H  S  G  V  P  S

                                  270                                 300
AGGTTCAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTAGCAACCTGGAGCAA
 R  F  S  G  S  G  S  G  T  D  Y  S  L  T  I  S  N  L  E  Q

                                  330                                 360
GAAGATATTGCCACTTACTTTTGCCAACAGGGTGATACGGTTCCTTGGACGTTCGGTGGA
 E  D  I  A  T  Y  F  C  Q  Q  G  D  T  V  P  W  T  F  G  G

                        381
GGCACCAAGCTGGAAATCAAA
 G  T  K  L  E  I  K
```

Figure 20

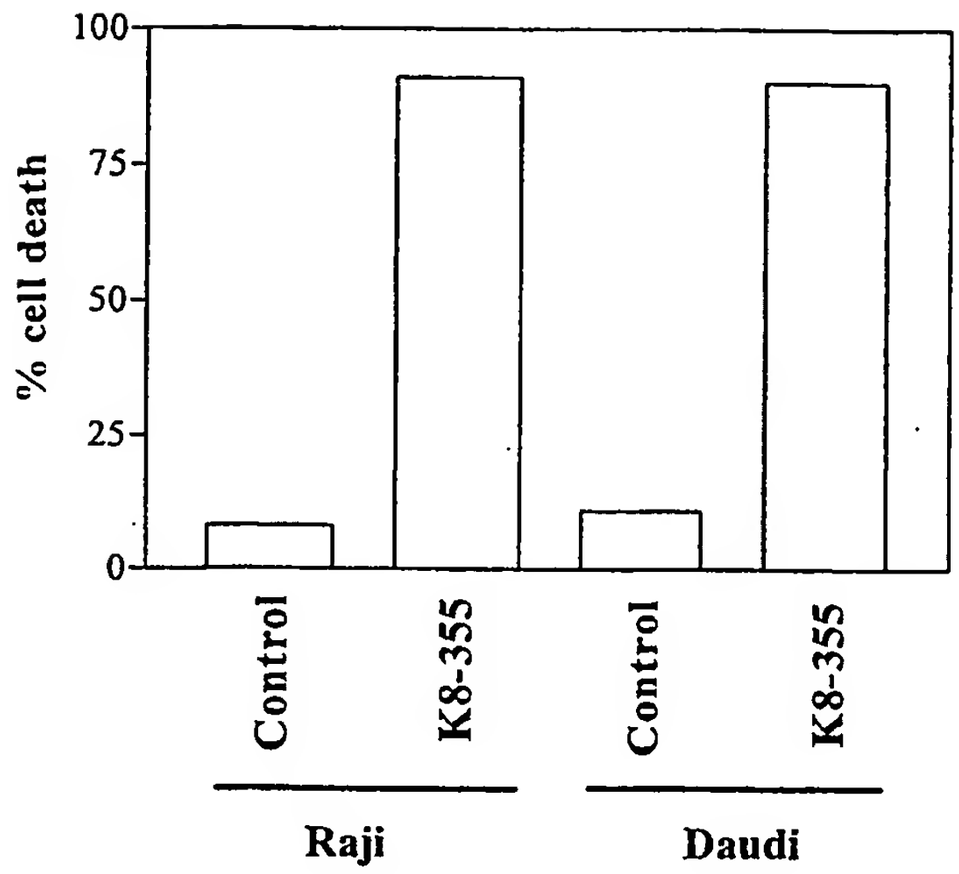

100
75
50
25
0
% cell death
Control
K8-355
Control
K8-355
Raji
Daudi

Figure 21

Table 1.

Sample number	*Identified Protein*	*Molecular mass (Da)*	*Theoretical pI*	*Accession Number*
5	ATP synthase, subunit beta	57955.9	5.14-5.8	179279, 13650291
9	prohibitin	29804.2	5.57	4505773
10	voltage-dependent anion channel 2	30412.3	6.81-7.5	12652825, 13645745
11	voltage-dependent anion channel 1	30727.7	8.62	6063691, 4507879
12	ATP synthase, subunit alpha	59750.9	8.95-9.16	4757810, 13111901

Figure 22

Table 2.

Clone #	Normal Prostate	Normal HUVEC	Prostate LNCaP	Prostate DU 145	Prostate PC-3	Pancreas PANC-1	Bladder RT4	Colon HT-29	Lung NCI-H292	Breast T-47D	Ovary NIH:OVCAR-3	Antigen MW (kD)
P1-42			++									98
P4-79			+++									100
P6-49			++									100
P9-32			++									100
P9-65			++									NA
P10-70			+++									NA
P10-82			+++									98
P12-22			+++									100
P2-23			+++							+/-		100
P10-2			+++							+/-		100
P10-62			++							+/-		98
P3-53			+++							++		120/110
P9-64			++							++		120/110
P10-28			++							+++		120/110
P10-29			++							+++		120/110
P8-2			+		+	++						NA
P8-11			+		+	++						NA
P8-14			+			++						NA
P8-35			+			++						NA
P8-74			++			++						NA

"+++" indicates more than 2 log of shift of FACS mean channel fluorescence compared to the negative control (secondary antibody only); "++" between 1 and 2 log, and "+" less than 1 log. "-" indicates no binding and "NA" means data not available.

Figure 23

Table 3.

Clone #	Normal Prostate	Normal Breast	Normal HUVEC	Prostate LNCaP	Prostate DU 145	Prostate PC-3	Pancreas PANC-1	Bladder RT4	Colon HT-29	Lung NCI-H292	Breast T-47D	Ovary OVCAR-3	Antigen MW (kD)
P11-65		+/-		++	++	++	+++	+	++	+	++	+	>220
P1-95				++			+	++	++	+	+	+	25
P2-68				+					+		+		40
P3-28				++				++	+				ND
P4-48				++		+	++				++		ND
P7-69				++			+						110
P8-20				++	+	+		++	+++	+	++	++	40
P8-32				+				+			+		28
P8-83				++		+	++		+		++	+/-	115
P11-49				+		+	+			+	+	+	ND
P11-57				+		+	++			++	+	+	ND
P11-85				+		+	+			+	++	+	60
P11-93				+	+	+	++		++		+ & ++	+/-	180
P12-27				+				+			+		ND

"+++" indicates more than 2 log of shift of FACS mean channel fluorescence compared to the negative control (secondary antibody only); "++" between 1 and 2 log, and "+" less than 1 log. "-" indicates no binding.

Figure 24

Table 4

Clone #	AML KG-1	AML THP-1	Granulocyte	Stem Cell +	Lymphocyte	Monocyte	Apoptosis KG-1	Apoptosis THP-1	Antigen MW (kD)
K1-34	+++	++	+/-	+++	+++	+	+	+++	28/32
K1-47	+++	++		++	+++	+++	++	+++	28/32
K1-79	+++	++		++	+++	+++	+	++	28/32
K1-95	+++	+++	+/-	++	++	+	+	+	28/32
K2-97	+++	++		+++	+++	+++	+		28/32
K2-124	+++	+++	+/-	+++	+++	+++	+		28/32
K2-167	++	+++		+++	+++	+++	+	+++	28/32
K5-37	+++	+++		+++	+++	+++	+		28/32
K6-98	+++	+++		+++	+++	+++	++	+++	28/32
K6-175	++	++		+	+	+	+++		28/32
K6-179	+++	+++	+/-	++	+/-	+	++	+++	28/32
K7-270	+++	+++		++	++	++	++	+++	28/32
K8-335	+++	++		++	+++	+++	+++	+++	28/32
K8-355	+++	+++		++	+++	+++	+++	++	28/32
K9-3	++	+++		+++	++	++	++	+++	28/32
K11-282	+++	++	++	++	+++	+++	+	+++	14
K12-328	++	+		++	+	+	+++	NA	40
K9-64	++	+		+++	+++	+++	++		32
K11-230	++	++		++	+	+		+++	180/80
K2-109	++	+		+	+	+	+	+++	ND
K2-127	++	+		++	+	+	+		ND
K5-71	+++			++	+	+			ND
K6-103	+++	++		+	++	+	++	NA	ND
K6-114	++	+		+	+++	+	++	++	ND
K6-121	++	+++		+	+	+	++	++	ND
K6-149	+++	++		+++	++	++	+	+++	ND
K6-150	++	+++		+++	+++	+++	+++	+++	30
K7-196	+++	++		+	+++	++	+	+++	ND
K7-275	++	+++		++	+	+		+++	ND
K8-343	+++	+++		++	+++	+++	+++	+++	ND
K8-364	++	+		+	++	+	+++	+++	ND
K8-365	+++	++		++	+++	+++	+++	+++	38
K9-92	++	+++		++	+	+	+	+++	80
K11-272	++	+	+/-	++	+++	+++	++	+++	97
K11-280	+++	+++	+/-	++	++	+	+	+++	80
K12-360	+++	+		++	++	+	+	+++	ND

Figure 25

SEQUENCE LISTING

<110> Tso, J.
Green, Jenniffer

<120> IDENTIFYING ANTI-TUMOR TARGETS OR AGENTS BY LIPID RAFT IMMUNIZATION AND PROTEOMICS

<130> 05882.0019.NPUS01

<150> USSN 06/329,178
<151> 2001-10-11

<150> USSN 60/331,965
<151> 2001-11-21

<160> 2

<170> PatentIn version 3.1

<210> 1
<211> 119
<212> PRT
<213> Mouse

<400> 1

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1 5 10 15

Thr Val Lys Ile Ser Cys Lys Ala Ser Lys Tyr Thr Phe Thr Asn Tyr
20 25 30

Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Val Leu Arg Trp Met
35 40 45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Asp Asp Phe
50 55 60

Lys Gly Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Tyr
65 70 75 80

Leu Gln Ile Asn Asn Leu Lys Asn Glu Asp Met Ala Thr Tyr Phe Cys
85 90 95

Ala Thr Thr Thr Leu Ile Thr Tyr Tyr Phe Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Thr Leu Thr Val Ser Ser
115

<210> 2
<211> 107
<212> PRT
<213> Mouse

<400> 2

Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu Gly
1 5 10 15

Asp Arg Val Thr Ile Ser Cys Arg Ser Ser Gln Asp Ile Ser Lys Tyr
20 25 30

Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys Leu Leu Ile
35 40 45

Tyr Tyr Thr Ser Arg Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Gln
65 70 75 80

Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln Gly Asp Thr Val Pro Trp
85 90 95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
100 105

FCE Events Calendar

09/14/2006

09/14/2006

INN-108	2nd extension, response to FINAL OA
G-085US05PCT	3rd extension, response to office action w/fee
G-077US03DIV	Timely response to office action
SER-106D1T	Timely response to office action

09/21/2006

09/21/2006

INN-114T	2nd extension, response to office action w/fee

09/22/2006

09/22/2006

G-036US04DIV	3rd extension, response to FINAL OA

09/25/2006

09/25/2006

INN-102	2nd extension, response to office action w/fee

09/27/2006

09/27/2006

INN-103TD2	Timely response to office action

09/29/2006

09/29/2006

G-056US04CIP	3rd extension, response to FINAL OA ABANDON

10/02/2006

10/02/2006

MDH-100XC1T	1st extension, response to FINAL OA
G-161US02PCT	1st extension, response to FINAL OA

10/03/2006

10/03/2006

G-085US04CON	2nd extension, response to FINAL OA

10/04/2006

10/04/2006

INN-112	3rd extension, response to office action w/fee

FCE Events Calendar

10/12/2006

10/12/2006

UF-288XC1	Timely response to office action

10/31/2006

10/31/2006

MET-037CXT	Timely response to office action

11/08/2006

11/08/2006

G-029US05DIV	Timely response to office action

11/09/2006

11/09/2006

DAS-120XC1	Timely response to office action

11/13/2006

11/13/2006

G-087US03PCT	Timely response to office action

11/24/2006

11/24/2006

G-194US02REG	Timely response to FINAL OA

11/28/2006

11/28/2006

G-091US10DIV	Timely response to office action

12/06/2006

12/06/2006

ARS-103	Timely response to FINAL OA
G-056US05DIV	Timely response to office action